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Study of Enrofloxacin Depletion in the Eggs of Laying Hens Using Diphasic Dialysis Extraction/Purification and Determinative HPLC-MS Analysis

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A study of the depletion of enrofloxacin residues in eggs was carried out using a diphasic dialysis procedure for the extraction of fluoroquinolone residues from the matrix. Enrofloxacin was administered to laying hens through the intramuscular route (15 mg/day) and orally (12 mg/day). After daily collection, the egg albumen and the egg yolk were separated, and the residue levels were determined using an HPLC-MS (API-ESI) method. The enrofloxacin and ciprofloxacin peaks gradually increased until the fifth day, because the drug was employed for 5 days. However, differences were observed in the depletion curves of enrofloxacin and its metabolite when both parts of the egg and the mode of administration were considered.

KEYWORDS: Fluoroquinolone; enrofloxacin; ciprofloxacin; diphasic dialysis; egg; LC-MS

INTRODUCTION

Owing to both their broad spectrum and their physicochemical properties, fluoroquinolones are some of the most useful antimicrobial agents used in human and animal medicine. The antimicrobial activity of the quinolones is related to the inhibition of bacterial DNA gyrase. Without the gyrase, DNA cannot be replicated and later repacked into daughter cells. The action of quinolones also inhibits the relaxation of the supercoiled (packed) DNA necessary for DNA replication, and it increases double-stranded DNA breakage (*1*). These antimicrobial agents are highly active against a broad range of bacteria, and hence their use is not restricted to human medicine but also finds wide application in the treatment and prevention of veterinary diseases in food-producing animals and even as growth-promoting agents (*2*).

Study of the use of compounds such as enrofloxacin, commonly used in the poultry industry for the control of early mortality, is a matter of special concern because this drug could contribute to the acquisition of resistance in bacteria (i.e., *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli*) and this, in turn, could lead to a reduction in the efficacy of such compounds in treating infections in humans. Furthermore, in the administration of drugs, residues or metabolites of these drugs may persist in food, causing evident concern with regard to human health issues (*2*).

In this regard, the maximum residue limits (MRL) for the fluoroquinolone enrofloxacin and its metabolite ciprofloxacin legally permitted in food under the laws of the European Union (*3*) have been established for the muscle, fat, liver, and milk from several animal species, but not for eggs. This is of special importance for egg producers so that a period of suppression of the drug can be established.

A review of the methods proposed for the analysis of quinolones in foods would include work by Hernández Arteseros et al. (*4*), Golet et al. (*5*), Roybal et al. (*6*), Donoghue and Schneider (*7*), Schneider and Donoghue (*8*), Kowalczuk et al. (*9*), and Liu et al. (*10*). Most of these methods involve a preliminary extraction step followed by a later cleanup step. Supercritical extraction has also been used for this purpose (*11*, *12*). In a previous work we developed a simple method for the analysis of enrofloxacin residues in eggs, with diphasic dialysis extraction and HPLC-MS detection (*13*).

The aim of the present work was to study the depletion curve of enrofloxacin and its metabolite ciprofloxacin in eggs (white and yolk) to help to establish the MRL for this residue in this foodstuff. To this end, here we studied the transfer and distribution of enrofloxacin and ciprofloxacin in the eggs of treated laying hens. Over a period of 30 days, each day we determined the residue levels of the drug in both yolk and albumen, determining the depletion curves of the two residual substances.

EXPERIMENTAL PROCEDURES

Dose. The pharmacological speciality Ganadexil (enrofloxacin 5%), obtained from Industrial Veterinaria, S.A. Invesa (Esplugues de Llobregat, Barcelona, Spain), was used.

Animals. Twelve laying hens housed in individual cages were used. Two of them were considered to be blanks, and of the others, five were dosed with enrofloxacin over 5 consecutive days at 15 mg/day

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through the intramuscular route and 5 were dosed with enrofloxacin at 12 mg/day orally in their water. Once treatment had begun, eggs were collected for daily analysis over 30 days. For the analyses, the yolk and white were separated and homogenized with an Ultra-Turrax T25 homogenizer (Staufen, Germany). The samples were analyzed every day.

Reagents and Chemicals. Standards of the enrofloxacin, ciprofloxacin, and lomefloxacin, HCl salts, were supplied by Bayer AG (Wuppertal, Germany). Acetonitrile, dichloromethane, isopropyl alcohol, and formic acid were purchased from Merck (Darmstadt, Germany). Citric acid and sodium citrate were obtained from Sigma-Aldrich (St. Louis, MO). Milli-Q organic-free water (Millipore, Bedford, MA) was used. All reagents were of analytical grade. Dialysis tubing was of type 20/32 (size 3), made of regenerated cellulose with a molecular exclusion size of 12−14 kDa from Visking, Medicell-International Ltd. (London, U.K.).

Standards. All standard solutions were prepared in 2% aqueous formic acid at a concentration of 100 mg mL^{-1}. These solutions were stored at 4 °C in the dark for no longer than 3 months. Standard working solutions were prepared freshly each day by dilution with water.

Preparation of Samples. The eggs were separated into whites and yolks, and these components were analyzed separately. Whites or yolks were homogenized with an Ultra-Turrax blender, and 5.00 g was mixed with 20 mL of citric acid/sodium citrate buffer, pH 6, with 1 mL of isopropyl alcohol, in an Erlenmeyer flask. Then, a previously wetted dialysis tubing made of regenerated cellulose containing 20 mL of dichloromethane with 1 mL of isopropyl alcohol was introduced into the Erlenmeyer flask. Extractions were performed by stirring at 150 rpm at 37 °C for 4.5 h in a shaker incubator, model G25&R25 from New Brunswick Scientific, Edison, NJ. The content of the dialysis tubing was then poured into a glass tube and evaporated to dryness under a nitrogen stream at 40 °C in a nitrogen evaporation system, with a thermostated heating plate from New Brunswick Scientific. The extracts were redissolved with 1 mL of 2% aqueous formic acid, mixed with 200 ng of lomefloxacin as internal standard, and injected into the HPLC-MS system.

Liquid Chromatography. The chromatographic system consisted of a Waters Alliance separation module (Milford, MA). Chromatographic separation was achieved on a C18 X-TerraTM MS column of 3.5 μm (150 mm × 2.1 mm i.d.) from Waters. The mobile phase consisted of a mixture of acetonitrile and water (10:90) adjusted to pH 2.8 with formic acid. The eluent was carefully degassed with helium and filtered prior to its use. A flow rate of 0.3 mL/min was applied.

Mass Spectrometry. The HPLC system was coupled to a Micromass Platform LC (Manchester, U.K.) mass spectrometer with an API-ES interface. The pseudo-molecular ion $[M + H]^+$, the loss of a CO_2 molecule $[M - CO_2 + H]^+$, and the adduct with acetonitrile $[M + MeCN + H]^+$ for each quinolone were monitored in the selected ion recording mode (SIR), with a dwell time of 0.06 s and an interchannel delay of 0.01 s. The pseudo-molecular ions were used for quantification. The MS instrument was operated in positive mode.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of an extract of egg yolk (day 4 after the first dose) from a hen treated by injection. The eggs (collected daily) from all of the treated hens were separated into white and yolk and analyzed following our previously published method, which uses a diphasic dialysis extraction procedure followed by quantitative HPLC-MS analysis (*13*). Ciprofloxacin and enrofloxacin concentrations in the samples were interpolated from the five-point calibration curves made with 5 g of whole egg described in the above-mentioned work. This method was designed for whole egg analysis. Prior its application in this work, we used five samples of white and yolk, at the levels of the five-point calibration curves, to verify (a) that the percentages were within the established range (70−104 and 55−97% for the drug and its metabolite, respectively) and (b) the similarity of the curves. A conventional Student's *t* test (*14*) was applied to compare the two curves (yolk and white)

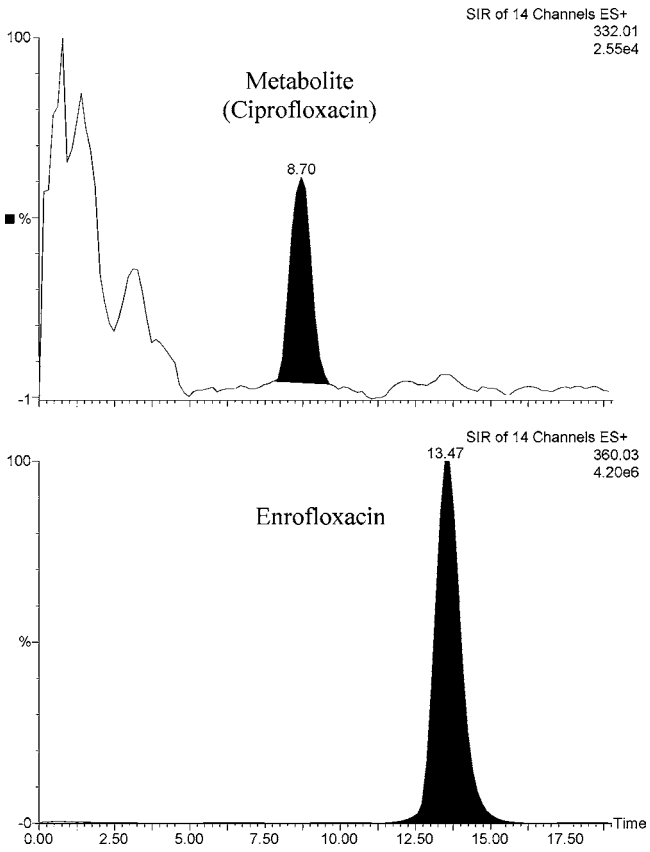

Figure 1. Chromatogram of a diphasic dialysis extract from egg sample containing ciprofloxacin and enrofloxacin obtained from an injected laying hen.

with the curve made with whole egg. We concluded that the efficiency of the extraction procedure using diphasic dialysis does not depend on the sample type, and the results obtained from whole egg could be extrapolated perfectly for the yolk and white samples separately.

When the drug administered reaches the bloodstream, it is distributed throughout the body. These compounds are in the ovary, growing follicles, and oviduct. Here the egg white is formed and secreted. The levels of residues of all the drugs, fluoroquinolones and their metabolites included, depend on physicochemical characteristics.

To understand the distribution of veterinary drug residues, it is essential to take into account the formation and composition of eggs. The water-soluble proteins of the white are formed in and secreted by the magnum of the oviduct. This process takes 1 or 2 days. This is why the time required to achieve maximum residue levels in the white is generally 2−3 days. The yolk is mainly formed of lipoproteins synthesized in the liver. Residues of drugs in the yolk generally required 8−10 days to reach maximum levels (*15*).

In the present work we treated the laying hens in a way similar to the situation on egg-producing farms. Intramuscular and oral administrations were assayed, although administration in water is the normal procedure for enrofloxacin treatment in laying hens. The other mode (intramuscular) was also studied because the pharmacological speciality Ganadexil is designed for this use.

Ciprofloxacin has been described as the main metabolite of enrofloxacin, with extensive metabolism in chickens (*16*). In light of this, here we report the results for both substances. **Tables 1** and **2** show the results in albumen and yolk, with the two modes of treatment. The recovery percentage used each

Table 1. Residues of Enrofloxacin in Albumen and Yolk of Eggs from Hens Receiving Enrofloxacin over 5 Consecutive Days in a Dose of 15 mg/Day through the Intramuscular Route or 12 mg/Day through Oral Consumption in Drinking Water

		albumen				yolk			
		injected		water		injected		water	
day	% recovery[a]	av, $n = 5$[b]	SD	av	SD	av, $n = 5$[b]	SD	av, $n = 5$[b]	SD
1	85	0.0	0	0.0	0	0.0	0	0.0	0
2	78	3928.8	580	5407.3	475	895.0	285	1407.3	500
3	85	5320.0	600	6484.2	675	1720.0	385	2186.7	277
4	95	4217.7	494	6295.4	590	1645.4	289	2935.7	652
5[c]	84	2719.6	483	5820.0	486	1291.6	208	2270.0	365
6	129	1200.1	411	4524.3	988	1021.0	181	2171.5	452
7	66	628.6	100	2965.9	1013	1037.8	259	1270.0	256
8	88	124.4	34	3148.0	800	720.0	0	1311.5	304
9	107	200.5	50	568.5	379	846.5	252	736.0	91
10	85	59.8	6	346.7	212	409.7	118	638.9	303
11	109	79.1	18	300.0	212	414.4	151	573.1	0
12	89	38.8	26	265.0	113	55.9	17	353.5	39
13	81	38.1	14	85.7	58	26.9	0	187.9	127
14	60	34.9	10	49.6	$n = 2$	21.3	$n = 1$[d]	no data	
15	62	33.5	9	20.4	$n = 1$[d]	no data		23.6	$n = 2$
16	84	26.9	$n = 2$	26.2	$n = 1$[d]	26.5	$n = 2$	77.0	$n = 1$[d]
17	99	7.5	$n = 1$[d]	21.7	$n = 1$[d]	20.7	$n = 1$[d]	19.0	$n = 1$[d]
18	83	6.0	$n = 1$[d]	no data		no data	$n = 1$[d]	51.0	$n = 1$[d]
19	79	4.7	$n = 1$[d]	17.3	$n = 1$[d]	20.6	$n = 1$[d]	12.3	$n = 1$[d]
20	75	2.3	$n = 1$[d]	6.4	$n = 1$[d]	28.1	$n = 1$[d]	3.5	$n = 1$[d]
21	80	2.0	$n = 1$[d]	<LOD[f]		15.5	$n = 1$[d]	<LOD	
22	98	<LOD[f]		<LOD[f]		9.3	$n = 1$[d]	<LOD	
23	84	<LOD[f]		<LOD[f]		<LOD[f]		<LOD	

[a] 10 ng g^{-1} of spiked amount. [b] $n = 5$, results from the 5 eggs collected each day. [c] Last day of treatment with enrofloxacin. [d] $n = 1$, only 1 egg positive from the 5 eggs collected this day. [e] Only 2 eggs positive from the 5 eggs collected this day. [f] <LOD, all 5 eggs collected this day have an enrofloxacin content of <2 ng g^{-1}.

Table 2. Residual Contents of the Metabolite Ciprofloxacin in Albumen and Yolk of Eggs from Hens Receiving Enrofloxacin over 5 Consecutive Days in a Dose of 15 mg/Day through the Intramuscular Route or 12 mg/Day through Oral Consumption in Drinking Water

		albumen				yolk			
		injected		water		injected		water	
day	% recovery[a]	av, $n = 5$[b]	SD	average	SD	av $n = 5$	SD	av	SD
1	72	0.0	0	0.0	0	0.0	0	0.0	0
2	80	56.1	20	93.7	36	3.9	2	31.5	14
3	68	58.7	22	129.7	36	13.4	0	63.1	23
4	84	95.8	25	119.0	41	59.7	22	94.7	25
5[c]	77	39.4	12	126.8	30	95.2	32	145.5	0
6	78	42.3	15	86.1	37	132.0	51	192.1	45
7	72	19.6	$n = 1$[d]	58.0	11	259.6	55	218.7	58
8	71	<LOD[e]		36.3	5	401.7	63	224.6	55
9	63	<LOD[e]		7.5	$n = 1$	331.8	65	267.2	71
10	72	<LOD[e]		1.5	$n = 1$	162.3	56	294.7	44
11	77	<LOD[e]		<LOD[e]		121.2	25	260.6	0
12	63	<LOD[e]		<LOD[e]		19.1	17	117.5	58
13	70	<LOD[e]		<LOD[e]		<LOD[e]		51.1	12
14	66	<LOD[e]		<LOD[e]		<LOD[e]		9.2	$n = 1$
15	66	<LOD[e]		<LOD[e]		<LOD[e]		4.5	$n = 1$
16	59	<LOD[e]		<LOD[e]		<LOD[e]		<LOD[e]	

[a] 20 ng g^{-1} of spiked amount. [b] $n = 5$, results from the 5 eggs collected each day. [c] Last day of treatment with enrofloxacin. [d] $n = 1$, only 1 egg positive from the 5 eggs collected this day. [e] <LOD, all 5 eggs collected this day have an enrofloxacin content of <4 ng g^{-1}.

day (shown in the tables) was obtained from a whole egg containing 2 and 4 ng/g of enrofloxacin and ciprofloxacin, respectively.

The first positive result was observed 48 h after the beginning of the treatment. In the egg of the first day no quinolones were present because it was already formed when the drug was administered to the hens.

Enrofloxacin levels were always ~100 times greater than those of its metabolite ciprofloxacin. This is in agreement with the findings of Gorla et al. (*17*), who also detected the metabolite in low levels in eggs from treated birds.

The enrofloxacin and ciprofloxacin peaks increased gradually until the fifth day, because the drug was employed for 5 days. However, differences were observed in the depletion curves of enrofloxacin and its metabolite when both parts of the egg and the mode of administration were considered.

Regarding distribution, Gorla et al. (*17*) referred to the liposolubility and physicochemical characteristics of drugs to explain the differences in quinolone levels in white and yolk. These authors also considered diffusion as a possibility to explain the observed distribution of enrofloxacin in yolk and white. Roudaut (*18*) considered protein binding to be a possible explanation for the observed distribution of oxolinic acid between white and yolk. In the present work, the maximum level of enrofloxacin residue was found in the white on the third day with both treatments. A stronger accumulation in the white

was observed, whereas the residues of this substance were less present in the yolk, in the same way. Gorla et al. (*17*) also found a similar distribution for enrofloxacin (white)/enrofloxacin (yolk) in the eggs of laying hens treated with 5 mg/kg/day over 5 days. After the last dose, the curve for enrofloxacin fell rapidly, whereas the metabolite ciprofloxacin remained for several days in the yolk, its maximum level being reached on the seventh day in injected hens and on the ninth day in laying hens receiving the drug in water. Enrofloxacin metabolism can be invoked to explain the distribution found. The liver is the organ in which ciprofloxacin synthesis takes place. It is also the organ that produces lipoproteins (the major constituents of yolk). Bearing this in mind, it is possible to understand the higher level of ciprofloxacin found in the yolk.

With water treatment, metabolite residues persist longer at levels close to the maximum, before diminishing on day 12 to amounts of <30 ng g^{-1}. A delay in the absorption of orally administered fluoroquinolone can account for these observations.

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