Enrofloxacin and Ciprofloxacin Residues in Table Eggs: Distribution and Heat Treatment Effect

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RESEARCH ARTICLE

Abstract

A 500 composite egg samples (2500 eggs-each sample represented by 5 eggs) collected from layer farms and local markets from all over Jordan were studied for presence of antimicrobials using Premi®Test screening test. Positive samples indicated by inhibition of microbial growth represented 12.8% out of total screened egg samples. Positive samples were examined quantitatively using HPLC technique to detect the presence of enrofloxacin and ciprofloxacin. Ciprofloxacin and enrofloxacin residues were detected in 1% and 0.8% of the total samples, respectively, where both drugs were recovered from white but not yolk. The effect of boiling on either drugs concentration in fortified white or yolk was demonstrated by gradual increases in the mean reduction percentages within treatment time with an average of 87% reduction after 15 minutes of boiling for both drugs and egg compartments and 5 minutes of frying at 160°C. The average concentration reduction percentages by the end of four weeks of refrigeration of fortified yolk and white were around 45 and 50% for enrofloxacin and ciprofloxacin respectively. The significance and mechanism of drug deposition and reduction during processing is being highlighted.

Keywords: Antimicrobial residues; egg; ciprofloxacin; enrofloxacin; processing.

INTRODUCTION

The considerable increases in egg demands have led to production of table eggs in an intensive rearing operation. This intensive production of eggs has led to an extensive use of antimicrobials for growth promoting purposes, performance enhancers and feed efficiency beside disease treatment and prophylaxis (Serratos et al., 2006). Fluoroquinolones constitute an expanding group of synthetic antibiotics, widely used in the treatment of infections in both human and veterinary medicine. A number of these drugs have been licensed to be administered in chicken farms for the prophylaxis and treatment of respiratory, renal and digestive infections in different regions of the world (Andersson and MacGowan, 2003; Martinez et al., 2006; Almashhadany, 2020). Fluoroquinolones have often been used for the treatment of *C. jejuni* and species of non-typhoidal *Salmonella* in humans (Luangtongkum et al., 2009). Enrofloxacin (ENRO) and its major metabolite ciprofloxacin (CIPRO) that exhibits biological activity similar to that of the parent compound are prohibited from extra-label use in USA and not approved in EU for use in laying hens because of their accumulation and long-term persistence in eggs and processed eggs (albumin and freeze-dried-eggs) (CIDRAP,

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2005; European Commission, 2010; Goetting et al., 2011). However, data from the literature indicated that there were incidents of enrofloxacin illegal use in laying hens' therapy (Gdybik-Sikorska et al., 2013). At the same time enrofloxacin found its uses in laying poultry industry in developing countries (Lolo et al., 2005; Zayerzadeh et al., 2011) and thus may lead to residues appearing in meat and eggs.

The most common cause of drug residues in food products is the failure to meet the withdrawal times (Al-mashhadany, 2019), improper use of licensed substances (Cornejo et al., 2012) and the improper health status that may affect the pharmacokinetics and drug metabolism (Mbodi et al., 2014; Chen et al., 2019). After administration to laying hen, antimicrobial residue or their metabolites will appear in one or both of egg components (white and yolk). Intestinal absorption of the drugs and transportation through blood is the responsible cause for deposition in yolk in the ovary or white in the oviduct. Physicochemical properties of the drugs and the physiology of the chicken and of egg formation will determine the amount of deposited drug (Donoghue and Myers, 2000; Al-Mashhadany et al., 2018). Drugs distribution in between the two egg compartments might be related to the higher affinity of the antimicrobials to be dissolved in water and/or to egg and drug pH considerations. Other factors like protein or fat binding properties might also play their roles in antimicrobials egg distribution. Egg white has a higher water content and no fat usually present in white, but both compartments are a good source of protein (Alaboudi, 2017).

When fluoroquinolones given orally or by intramuscular injection to laying hens, their residues appear in eggs around 24 h after the first dose and persist in both yolk and albumin for several days after cessation of treatment (Lolo et al., 2005; Herranz et al., 2007). The reason for the different distribution in egg white and yolk of the fluoroquinolones may be related to the differences in lipo-solubility and physicochemical characteristics of the drugs, such as their molecular weight, pK_a value and binding capacity to albumin or plasma proteins (Kan and Petz, 2000). Although there is no general agreement regarding fluoroquinolone distribution, some authors described that these drugs accumulate mainly in the yolk, being deposited and incorporated to this matrix during the egg development (Donoghue and Myers, 2000; Bilandžić et al., 2015). However, other authors suggested that fluoroquinolone residue contents are higher in egg white (Gorla et al., 1997; Kan and Petz, 2000).

On a residue analysis study on laying hens; after 5 days treatment with enrofloxacin and ciprofloxacin in a dose of 5 mg/kg body weight per day, the detected concentration in egg white and yolk were, during 5 days ranged from 0.02 to 1.98 $\mu\text{g/g}$ (enrofloxacin) or 0.14 to 0.28 $\mu\text{g/g}$ (ciprofloxacin) respectively (Gorla et al., 1997). Also, Ciprofloxacin was detectable in egg white on the first day of treatment (orally administered 10 mg/kg ciprofloxacin for five consecutive days) in higher concentrations (1755 $\mu\text{g/kg}$) while at lower concentrations (362 $\mu\text{g/kg}$) in egg yolk. In both white and yolk, concentrations increased during a five days treatment period. After withdrawal of treatment, eight days and fourteen days were required to deplete the drug residue below the established LOD in albumen and yolk respectively (Billah et al., 2015).

It was recommended that in egg, a withdrawal time of 10 days following 5-day administration of ENRO, based on the time needed for residue levels to decline below the level of detection (0.5 mg/kg) (Cornejo et al., 2012). Also, it was reported that ENR and CIP residues were detectable in both white and yolk for 15 days after drug withdrawal in concentrations of 6.4 and 3.5 $\mu\text{g/kg}$, respectively (Lolo et al., 2005). Table eggs are mostly refrigerated or exposed to heat treatment before being consumed. Limited studies have been reported on the stability; structural changes or the causes of decreases in parent drug during different food processing (Lolo et al., 2006; Okerman et al., 2007).

Considering the above-mentioned egg safety issues and the fact that conflicting data presented on few published information regarding ENR distribution in eggs, this study was designed to shed light on this issue. The study designed investigating the prevalence and distribution in between yolk and white of a commonly locally in use antimicrobials enrofloxacin or ciprofloxacin. Because of very little specific information on the importance of the effect of common egg heat treatment (boiling; frying), refrigeration and the stability of these drugs in egg compartments after processing were also investigated.

MATERIALS AND METHODS

Sample collection, transport, and preparation

Samples were collected from both the layer farms and the local market from all over Jordanian governorates in proportional to the number of table eggs produced in that governorate. Multi stage random sampling technique was used to select 140 layers' farms and 80 retail markets distributed in Jordan. In the second stage, a cross-sectional study using a simple and systematic random sampling method, where eggs from each selected farm ($N = 30$ eggs per farm) and retail market ($N = 10$ eggs per market) were collected. Eggs' age was ranging between 3-5 days and were not washed and except for refrigeration were not processed in any forms. Eggs were transported in a cool ice box to the laboratory, kept chilled at 0-4°C for 5-7 days before being tested for the presence of antimicrobials. Fifteen eggs from each farm sample were chosen and divided into 3 pooled samples each of 5 eggs. From retail samples 5 eggs were selected and were pooled to form one composite sample. Therefore, a total of 500 composite samples (2500 eggs) were screened for the presence of antimicrobial residues.

Antimicrobial residues screening assay using Premi Test

Screening assay using Premi ampoules (DSM, Netherlands) was used. Sample preparation and working procedures were done as per manufacturer's instruction. Positive samples (no bacterial growth) indicated by no color change were kept frozen at -80°C until analyzed quantitatively and qualitatively by chromatographic technique.

Quantification and identification of enrofloxacin and ciprofloxacin residues using HPLC Chemicals and reagents

Enrofloxacin and ciprofloxacin standard 99.5% purity (USP, USA), acetonitrile, Hexane, water and methanol were HPLC grade (SDS, France), Tris (hydroxymethyl) aminomethane (Searle, England), Hydrochloric acid fuming (GCC, UK), Phosphoric acid (Baker, Phillipsburg), Triethylamine (Janssen, Belgium), 0.45 µm disposable syringe filter unit (MFS, USA) and SPE Cartridge (6 ml, 500 mg, C18; Supelco, USA). All solvents for HPLC application were filtered before use with the 0.45 µm porosity filter paper (Millipore) and degassed by ultrasonication for 30 minutes before use.

Sample preparation

According to a published method (Yorke and Froc, 2000), one and half grams of accurately weighed and homogenized samples were placed into 10 ml glass centrifuge tubes with a 900 µl of Tris-buffer pH 9.1. The total volume was stirred by a vortex mixer for 15 minutes and let in contact for 15 minutes. Then a volume of 3 ml of acetonitrile HPLC grade were added to the tube that has been stirred for 1 min followed by centrifugation for 3 minutes at 17000 rpm with the temperature set at 5°C. The supernatant was poured into second tube which is evaporated at 50°C under a nitrogen stream to near dryness. The content was reconstituted with 1.5 ml of the HPLC mobile phase (2.45g/l H₃PO₄-acetonitrile (87: 13, v: v) (British Pharmacopoeia Commission, 2011). Volume of 900 µl of HPLC grade hexane was added to the 1.5 ml reconstituted sample as a defatting step prior to the HPLC running. The extract was centrifuged for 3 minutes at 5, 100 g at 5°C, then the aqueous phase was filtered Using disposable syringe filter unit (0.45µm porosity) then analyzed by HPLC.

Preparation of standard calibration curves

Standard calibration curves were done for every HPLC sequence run by adding 50, 100, 250, 500, 1000 and 2000 µL of 100 µg/mL of combined solution of enrofloxacin and ciprofloxacin to 100 gm of white or yolk samples to give final concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 and 2 µg/g.

HPLC working conditions

The analyses were carried out on a Shimadzu Class VPHPLC system (Tokyo, Japan) equipped with SCL 10 A VP system controller, SPD-10 AD VP UV-Visible detector and LC 10 AD VP solvent delivery with SIL-10 AD VP auto injector. The column used was thermo-hypersil C18: 250 mm×4.6 mm i.d., 5 µm end capped column, with Phosphoric acid (2.45 g/L H₃PO₄), Acetonitrile (87+13, v/v) as a mobile phase at flow rate of 1.5 ml/min AUV-detector with a wavelength of 278 nm and injection volume of 50 µL was applied.

HPLC validation

Methods for enrofloxacin and ciprofloxacin were validated for egg white and yolk (Pecorelli et al., 2004). Parameters included in the validation were specificity, accuracy, precision, linearity, sensitivity, lower limit of detection and recovery. The accuracy values for the technique followed ranged from 86.7 to 93.9 (Table 1). The specificity of the technique was assured as no interfering peaks appeared in the chromatograph corresponding to retention times of either enrofloxacin or ciprofloxacin in both yolk and white. The intra- or inter-day coefficients of variation for enrofloxacin and ciprofloxacin ranged between 0.3-12% (below 15%) at concentrations of 0.2, 0.8 and 1.6 mg/g.

Table 1. Summary of values of the measured parameters for method validation

Drug	Ciprofloxacin		Enrofloxacin	
Parameter	White	Yolk	White	Yolk
Accuracy %	86.7	90.4	93.9	89.5
CV %	0.6-12	0.3-11.6	0.9-9.3	0.4-9.2
Recovery %	89.5	87.6	91	86.7
LOD (µg/gm)	0.05	0.05	0.05	0.05
r ²	0.998	0.995	0.999	0.999

CV% = Coefficient of variation; LOD = Limits of detection; r² = Correlation coefficient.

The recoveries of the two antimicrobial drugs from both egg white and egg yolk ranged from 86.7% to 91%. The area under the curve was proportionally related to the antimicrobial drugs concentrations with correlation coefficient (r^2) values ranging from 0.995 to 0.999 indicating the linearity of the HPLC method. The measured quantitative lower limit of detection for the two drugs was (0.05 µg/g)

Heat treatment effect

Three samples of approximately 150 g of either yolk or white were fortified with a known amount of antibiotics (see standard egg preparation) and divided into approximately 3 equal portions of 50 g. For boiling, samples were placed in a 50 ml polyethylene screw capped tube and subjected to boiling in a controlled temperature water bath adjusted at 100 °C for up to 15 minutes. The temperature achieved in the sample during heat treatment procedure was monitored using an electronic digital thermometer (Huger, Germany) that has been inserted in the center of the sample. Both quantified egg whites and yolks were subjected to frying in olive oil at 160°C for 5 minutes. Samples were fried in a frying pan. The temperature achieved in the sample during heat treatment procedure was monitored using an electronic digital thermometer that has been inserted in the center of the sample. Portion samples were taken after 1, 3 and 5 minutes during the boiling or frying process, and cooled immediately to be analyzed for the presence and concentrations of antibacterial drug residues. Refrigeration was done at 8-10 °C for 4 weeks. Calculated results for all treatments were compared and analyzed statistically.

Statistical analysis

Statistical analysis was performed using the SAS software package, through which ANOVA test was used (t-test, multiple comparisons LSD, Fisher). The differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSIONS

Antimicrobial residues screening assay using Premi Test.

Sixty-four out of 500 (12.8%) of composite egg samples showed the presence of antimicrobial residues. Antimicrobials were detected in either 44 egg white, 17 yolk or in both compartments within 9 samples. Therefore, egg white characterized by the highest prevalence rate of 8.8% which differs significantly ($P < 0.05$) from that of yolk (3.4%) or the combination of both egg yolk and white (1.8%).

This study confirmed the presence of antimicrobial drug residues of both enrofloxacin and its body metabolite ciprofloxacin in table eggs produced and marketed in Jordan. Both drugs were detected from white but not from yolk. Lolo and associates reported that enrofloxacin and ciprofloxacin residues were detectable in both white and yolk for 15 days after drug withdrawal in concentrations of 6.4 and 3.5 µg / kg, respectively (Lolo et al., 2005). Other studies showed results for enrofloxacin residues are with a 6-8days depletion time for albumen and 9-10 days depletion time for yolk (Gorla et al., 1997; Cornejo et al., 2012). There are always no-good explanations that could be given to combined complicated factors that determine the distribution of a drug between egg white and yolk (Alaboudi et al., 2013). Some explanations to this complicated issue have suggested that egg yolk and egg white are not two phases separated by a semi-permeable membrane and in some way in equilibrium with each other for certain chemicals (Kan and Petz, 2000).

Diffusion might be a possibility to explain the observed distribution of between yolk and egg white (Gorla et al., 1997). However generally, three good reasons might rule the distribution of drugs between egg yolk and egg white: lipid solubility as fat soluble compounds generally occur in yolk, pK_a value as ionized molecules will distribute in a certain way between phases with different pH values such as yolk and white and thirdly protein binding of drugs to egg white proteins (Kan and Petz, 2000). The pH of egg white in fresh egg is 7.6-7.9 but in yolk it is only 6.0, which enhances the dissolution of antimicrobial drugs that have lower pH value. Both ciprofloxacin and enrofloxacin are generally with slightly acidic pH (5.5) (Furusawa, 2001). At the same time high white levels may suggest that quinolones have special characteristics in combination with egg white (proteins). This is besides that ciprofloxacin as a metabolite of enrofloxacin and, thus generally have better solubility in higher water contents of egg white. These conflicting data might necessarily need good reasonable verification.

Quantification of ciprofloxacin and enrofloxacin residues in table egg

In all egg samples examined, ciprofloxacin and enrofloxacin residues were detected in 5 (0.1%) and 4 (0.08%) samples respectively and both antimicrobials were recovered from white but not yolk (Table 1). All positive samples contained the enrofloxacin residue at concentration higher than the recommended MRL set by European Agency (The European Agency for the Evaluation of Medicinal Products, 2002). Enrofloxacin and ciprofloxacin were classified as unauthorized substances for use in animals from which eggs are produced for human consumption and no MRL was set for both compounds in egg whereas, only 100 µg/g was set as MRL for poultry tissue by ECC - Council Regulation (EEC - Council Regulation, 2006; European Commission, 2009). There was a significant

difference ($P < 0.05$) between the mean residual concentration of (355 $\mu\text{g/g}$) for enrofloxacin and the 139 $\mu\text{g/g}$ of the ciprofloxacin.

Table 2. Quantification of ciprofloxacin and enrofloxacin residues ($\mu\text{g/g}$) in table eggs

Ciprofloxacin	Enrofloxacin
340 ^a	1040 ^a
65 ^a	223 ^a
65 ^a	192 ^a
140 ^a	-
87 ^a	320 ^a
Mean $\pm$ SD=139 $\pm$ 116.3	Mean $\pm$ SD =355 $\pm$ 400.2*

^a Higher than MRL (100 $\mu\text{g/kg}$ for poultry tissue by ECC - Council Regulation (EEC - Council Regulation, 2006; European Commission, 2010).

*Significant differences between the two residues.

Although these prevalence rates are considerably low, however their presence still forms public health significance, as both these two drugs are in use in human medicine. This is beside that, the high detected concentration (above MRL) in egg of these drug is a good indication of the misuse of these drugs in laying hens. The MRL permitted by the European Agency for evaluating the medical products, Committee for veterinary medicinal products for enrofloxacin and its metabolite ciprofloxacin is only 100 $\mu\text{g/kg}$ in broiler muscles but no residues are permitted in in animals from which egg eggs are produced for human consumption (European Commission, 2009).

Effect of boiling on ciprofloxacin and enrofloxacin residues

Gradual increases in the mean reduction percentages within treatment time were observed in white or yolk with an average of 87% reduction after 15 minutes of boiling (Table 3). There was a significant difference in the reduction percentage of the mean residual concentration in between the three-time intervals ($P < 0.05$) No significant difference was calculated in between the white and yolk final reduction percentages.

Table 3. Effect of boiling on ciprofloxacin residues concentrations mean in egg white and yolk

Time (min)	Reduction % of enrofloxacin		Reduction % of ciprofloxacin	
	in white	in yolk	in white	in yolk
5	45 $\pm$ 0.3	39 $\pm$ 0.5	41.1 $\pm$ 0.3	37 $\pm$ 0.3
10	66.7 $\pm$ 0.2	60 $\pm$ 1.0	62 $\pm$ 0.5	51.5 $\pm$ 0.2
15	89 $\pm$ 0.1	86.7 $\pm$ 0.6	86.8 $\pm$ 0.3	85.6 $\pm$ 0.4

Effect of boiling on ciprofloxacin and enrofloxacin residues

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Effect of frying on ciprofloxacin and enrofloxacin residues

The reduction levels for both antimicrobials in both egg compartments (yolk and white) were time dependent. The longer the time, the higher were the calculated reduction percentages. After 5 minutes of treatment, the average reduction percentages were 94 and 93 in ciprofloxacin or enrofloxacin concentrations in white and yolk respectively (Table 4). There were significant differences between the reduction percentages of the mean residual concentration during the three-time intervals ($P < 0.05$) in both yolk and white and for both antimicrobials, but no significant difference was calculated in between the white and yolk final reduction percentages.

Table 4. Effect of frying on the average % of reduction of enrofloxacin and ciprofloxacin residue's concentration in egg white and yolk

Time (min)	Reduction % of enrofloxacin in		Reduction % of ciprofloxacin in	
	white	yolk	white	yolk
1	65 $\pm$ 1.0	63.7 $\pm$ 0.4	65 $\pm$ 1.0	63.1 $\pm$ 0.6
3	87.2 $\pm$ 0.9	86.2 $\pm$ 0.4	80 $\pm$ 0.7	83.3 $\pm$ 0.4
5	93.5 $\pm$ 0.4	92.9 $\pm$ 0.4	93.5 $\pm$ 0.5	92.9 $\pm$ 0.4

Effect of refrigeration on ciprofloxacin and enrofloxacin residues

Again, the longer the refrigeration intervals the higher the reduction percentages in concentrations of both antimicrobials under study in either yolk or white. The average concentration reduction percentages by the end of four weeks of refrigeration were around 45 and 50% for enrofloxacin and ciprofloxacin respectively. The difference in the reduction percentage of the mean residual concentration was significant between the four time intervals ($P < 0.05$) for both antimicrobials and compartments. No significant difference was calculated in between the white and yolk final reduction percentages (Table 5).

Table 5. Effect of refrigeration on ciprofloxacin and enrofloxacin residues

Time (week)	Reduction % of enrofloxacin in		Reduction % of ciprofloxacin in	
	white	yolk	white	yolk
1	12 ± 0.9	13 ± 0.5	17.1 ± 0.3	15 ± 1.0
2	20 ± 0.9	22 ± 0.9	25 ± 0.4	26 ± 1.0
3	36 ± 0.9	34 ± 0.9	32 ± 0.9	38.3 ± 4.0
4	46 ± 1.0	45 ± 0.4	50 ± 2.9	52 ± 2.0

Few studies have been reported on occurrence of drug residues in cooked food, nor on the stability of these compounds following heat treatment (Rose et al., 1996). In water enrofloxacin remain stable for 3 hours when heated at 100°C and poultry cooking (microwaving, roasting, boiling, grilling and frying) did not affect the enrofloxacin residues as they remain stable during heating (Lolo et al., 2006). However, in this study and after boiling at 100 °C for 15 minutes or frying at 160 °C for 5 minutes, both understudy drugs showed a significant reduction percentage in the mean residual concentration in both white and yolk indicating a high sensitivity of the drugs for the high temperature treatments. The two antimicrobial drugs were also unstable under the refrigeration conditions at 10°C or storage at 28-30°C. This instability was obvious from the 50% percentages of reduction in drugs level after one month of refrigeration or the average of 48% reduction after two weeks' storage at 28 °C.

CONCLUSIONS

Veterinary drugs are therapeutically used for laying hens but may also reach them unintentionally via the feed e.g. as a result of cross-contamination during premix manufacture, feed preparation in the feed mill or during feed transport. The mere presence of antimicrobials in poultry products reassure our expectations in the bad husbandry practices in studied farms and the failure to meet the withdrawal times or the extra-dosage regimen practiced in poultry rearing farms also might play a good role in prevalence of drug residues too. It was concluded that when enrofloxacin or enrofloxacin residues are suspected in egg, egg white is the preferred matrix for residue monitoring. High heat treatment temperatures for enough periods are effective in decreasing the levels of antimicrobial drug residues to low levels. However, storage of table egg at refrigeration at 10° C does not necessarily reduce the level of the residues to safe acceptable concentrations.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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