Withdrawal Times of Oxytetracycline and Tylosin in Eggs of Laying Hens after Oral Administration

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ABSTRACT

Antimicrobials administered to laying hens may be distributed into egg white or yolk, indicating the importance of evaluating withdrawal times (WDTs) of the pharmaceutical formulations. In the present study, oxytetracycline and tylosin’s WDTs were estimated. The concentration and depletion of these molecules in eggs were linked to their pharmacokinetic and physicochemical properties. Twenty-seven Leghorn hens were used: 12 treated with oxytetracycline, 12 treated with tylosin, and 3 remained as an untreated control group. After completion of therapies, eggs were collected daily and drug concentrations in egg white and yolk were assessed. The yolk was used as the target tissue to evaluate the WDT; the results were 9 and 3 days for oxytetracycline and tylosin, respectively. In particular, oxytetracycline has a good oral bioavailability, a moderate apparent volume of distribution, a molecular weight of 460 g/mol, and is lightly liposoluble. Tylosin, a hydrosoluble compound, with a molecular weight of 916 g/mol, has a low oral bioavailability and a low apparent volume of distribution, too. Present results suggest that the WDTs of the studied antimicrobials are strongly influenced by their oral bioavailability, the distribution, and the molecular weight and solubility, and that these properties also influence the distribution between the egg yolk and white.

Nowadays, good practices in the use of antimicrobial agents in food-producing animals has become an issue of special concern, aiming to reduce or prevent antimicrobial resistance and avoid the presence of these drug residues in food for human consumption (6). In the case of poultry, the antimicrobial agents are the main therapeutic tool used for the control of bacterial diseases (11). Therapeutic doses of these drugs administered to laying hens can lead to the presence of the parent drug and/or its metabolites in eggs. To ensure the safety of these products, only eggs produced after completion of an adequate withdrawal period can be used for human consumption (24). Thus, many authors have studied and established the withdrawal times (WDTs) for several veterinary antimicrobials in eggs, considering the egg as a whole edible product (4, 8, 27, 31) or egg compartments considered as separate matrices (9, 27, 32, 33). Different authors have studied factors that determine the antimicrobial agents’ distribution between egg yolk and white; however, currently, there is no consensus.

It has been stated that drug concentration in each egg compartment (yolk and white), mostly depends upon the physicochemical properties of the drug, such as the molecular weight and solubility. Different authors emphasize that water-soluble drugs have a higher affinity for the egg white, while the yolk has a higher concentration of lipid-soluble drugs (2, 20, 21), thus defining the pharmacokinetic behavior of the drug and, consequently, its presence in the different eggs components. Other researchers conclude that the main factor that determines the drug presence in egg yolk or white is the egg-forming physiology (14). According to these authors, the lipid-soluble antimicrobial agents can be detected for longer periods of time after therapy withdrawal, consequently producing longer WDTs than water-soluble antimicrobial agents. Some other authors point out that the distribution of the antimicrobial agents to egg components is a multifactorial phenomenon, which can also involve animal physiology factors, such as organ perfusion, tissue composition, and pH, among others. This complex mix of factors makes it difficult to predict the behavior of drug residues in eggs (12, 24, 25, 32).

Tylosin, a macrolide antibiotic, and oxytetracycline, from the tetracycline family, are two antibiotics used in poultry for disease treatment. Even though both drugs are widely used, they differ in their action range and in their pharmacokinetic and physicochemical characteristics. Oxytetracycline has a molecular weight of 460 g/mol, has a mild liposolubility, and presents a moderate oral bioavailability, with an intermediate apparent volume of distribution (1, 3). In contrast, tylosin has a molecular weight of 916 g/mol, is hydrosoluble, has a low oral bioavailability, and its apparent volume of distribution is low in comparison to oxytetracycline (23, 26).

According to the safety assessment of tylosin and oxytetracycline residues in eggs, the Codex Alimentarius (7)
assigned a maximum residue limit (MRL) of 400 µg/kg to oxytetracycline and a MRL to tylosin of 300 µg/kg in eggs. However, the European Union (17) has set a MRL of 200 µg/kg for both antimicrobial agents in this product.

Even when the MRLs for these antimicrobial agents are known, the available information in the literature regarding the distribution of these drugs in egg yolk and white and the WDTs for these drugs in eggs before being available for human consumption is limited. The goal of the present study was to evaluate the depletion of two antibiotics from different families, oxytetracycline and tylosin, in egg white and yolk and to compare the behavior of these two antimicrobials in both eggs compartments. For this purpose, the concentrations of these drugs in egg white and yolk were evaluated after therapy withdrawal. Following Regulation 37/2010/EC (17) recommendations, the concentrations of the parent compound oxytetracycline and its 4-epimer were assessed in the case of oxytetracycline depletion. For tylosin, the concentrations of the metabolite tylosin A were determined.

MATERIALS AND METHODS

Facilities. The present study was conducted in the Laboratory of Veterinary Pharmacology of the Veterinary and Animal Sciences Faculty, University of Chile, accredited under the International Organization for Standardization 17025 standards.

Pharmaceutical formulations. Two commercially available formulations were used: a pharmaceutical formulation of hydrochloride oxytetracycline 80% powder for the oxytetracycline study and a 10% powder formulation for the tylosin experiment. Prior to the study, the content of oxytetracycline and tylosin were verified in the solutions. For this purpose, standard calibration curves of certified standards of these drugs were performed.

Animals. Twenty-seven 20-week-old Leghorn hens were used; none of the hens had previous exposure to oxytetracycline and tylosin. This was confirmed by the chromatographic analysis of white and yolk, demonstrating the absence of drugs in eggs. Animals were maintained in batteries (25 ± 5°C temperature, 50 to 60% relative humidity) with ad libitum access to water and no medicated feed.

The diet was formulated according to the requirements outlined by the National Research Council (28), and the ingredients were previously analyzed to ensure antimicrobial agent absence. Experimental animals were kept in conditions in agreement with animal welfare guidelines outlined by the Bioethics Committee of the Veterinary Sciences Faculty of the University of Chile and recommendations of the European Council Directive 2007/43 (18).

Hens were randomly allocated in one of three experimental groups: group A and group B (n = 12) were treated with 40 mg/kg of body weight of oxytetracycline for 10 days and 35 mg/kg of body weight of tylosin for 7 days, respectively. Group C (n = 3) remained as untreated control hens. Treatments were given via a gastric tube to ensure total dose intake. The number of animals per treated group was selected according to the European Union standards for WDTs, established by the European Agency for the Evaluation of Medicinal Products (15), to obtain at least 10 eggs per sampling time. When the drug administration period was completed, eggs were collected daily for a 15-day period, labeled, and stored at −20°C until analysis.

Chemical reagents and standards. Oxytetracycline and tylosin purity standards were used, provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). The analytical grade citric acid, high-performance liquid chromatography (HPLC) grade water, analytical grade anhydrous sodium sulfate, HPLC grade acetonitrile, and analytical grade oxalic acid were purchased from Merck (Darmstadt, Germany). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ).

Standard and work solutions. Standard solutions of oxytetracycline were prepared in aqueous mobile phase at 4,000 ng/ml and standard solutions of tylosin in HPLC grade water at 10,000 ng/ml. Both solutions were 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. Eggs were analyzed individually each day. The egg white and egg yolk were separated for analysis of the components. Oxytetracycline extraction was carried out according to the method described by Cristofani et al. (10). Briefly, 10 g of yolk or white was homogenized, and 20 ml of McIlvaine-EDTA buffer (pH 4 ± 0.1) was added. The sample was vortexed for 1 min, sonicated for 5 min, and centrifuged at 3,130 × g for 15 min. The supernatant was filtered through glass wool. A solid-phase extraction column Sep-Pak C18 (Waters Corporation, Milford, MA) was used for the clean-up process, and elution was performed with 0.01 M oxalic acid in methanol. The fluid was dried under nitrogen stream at 40 ± 0.5°C, and the residue was dissolved with 500 µl of mobile phase. An aliquot of 100 µl was injected into the chromatographic system. The detection limit was set at 30 µg/kg in both egg components.

Tylosin extraction was carried out according to the method described by Civitareale et al. (5). Milliliters of phosphate buffer (pH 2.5), 5 ml of methanol, and 15 ml of HPLC grade water were added to 5 g of egg yolk or white. Samples were agitated for 5 min, sonicated for 5 min, and centrifuged at 3,130 × g for 10 min. The supernatant was drawn through a SCX Aromatic Sulfonic Acid extraction column (Bakerbond spe, Deventer, The Netherlands). The extraction columns were set up with 5 ml of methanol and 5 ml of phosphate buffer (pH 4.0). The elution was carried out with 2 ml of methanol. Each elute fraction was collected and dried under a stream of nitrogen at 45 ± 0.5°C. The residue was dissolved with 100 µl of mobile phase and injected into chromatographic system. The detection limit was set at 100 µg/kg in both egg compartments.

Liquid chromatography. Both antimicrobial agents were analyzed using an Elite LaChrom HPLC system coupled to a diode array detector (DAD; Hitachi High Technologies America, Inc.) operated at a wavelength of 360 nm. An X Terra RP18 analytical column (inside diameter, 100 by 4.6 mm) was used for oxytetracycline chromatographic separation. The mobile phase consisted of a mixture of 13% acetonitrile and 87% oxalic acid. The chromatography was conducted at 35°C at a flow rate of 0.7 ml/min and at wavelength of 360 nm.

A Chromolith RP18 analytical column (100 by 4.6 mm) and a Chromolith Guard Cartridge precolumn (Merck, Darmstadt, Germany) were used for tylosin chromatographic separation. The mobile phase was a mixture of 19% acetonitrile, 19% water, and 62% 0.02 M dibutyl ammonium phosphate solution. Chromatography was conducted at 30°C, with a flow rate of 1.0 ml/min and at wavelength of 287 nm.

Determination of WDTs. Oxytetracycline and tylosin concentrations in the incurred samples were calculated using the
equation from the regression analysis of the matrix-matched calibration curves (\( r > 0.97 \)) at different concentrations to avoid extrapolations.

The recommendations of the Evaluation of Medicinal Products (15) and the MRL of the European Union were considered to determine the WDT. 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**

Prior to the WDT calculation for each antimicrobial agent, the selected analytical methods were validated in both egg components, white and yolk, to demonstrate that the methods performance criteria were suitable for the detection of the target compounds in the selected component. For oxytetracycline, from day 1 to day 5 posttreatment, concentrations above the MRL in the egg yolk (200 \( \mu \)g/kg) were found. From day 1 posttreatment, concentrations were below the MRL in the egg white. Results are shown in Table 1. For tylosin, from day 1 posttreatment, concentrations in egg yolk and white were below the set MRL (200 \( \mu \)g/kg). Results are shown in Table 1.

The matrix-matched calibration curves were used for drug quantification in experimental samples. The linear regression analyses of the yolk depletion are shown in Figure 1. Considering the MRLs and the guidelines of the Evaluation of Medicinal Products (15), the WDT for oxytetracycline was determined to be 9 days. In the case of tylosin, even when the concentrations on the first day posttreatment were below the MRL, the WDT was 3 days when the 95\% upper one-sided tolerance limit was below the MRL with 95\% confidence.

**DISCUSSION**

Following the Codex Alimentarius recommendations (6), analytical methods for validation and calibration of equipment involved in the study are necessary for guaranteeing the quality of the laboratory results. Prior to the study, the analytical methods selected to quantify oxytetracycline and tylosin concentrations in egg yolk and white were validated following European Commission Decision 2002/657/EC guidelines (16), and all validation parameters met the criteria set in this guideline. These analytical methods have been previously used by other authors to determine drug residues in eggs (5, 10, 21).

According to the obtained results, egg yolk was considered the target tissue for the estimation of oxytetracycline and tylosin WDTs. The selection of yolk as target tissue for the studied drugs was decided because both

**TABLE 1. Daily average concentrations of oxytetracycline and tylosin in egg yolk and white after completion of treatment in laying hens**

<table>
<thead>
<tr>
<th>Posttreatment days</th>
<th>Oxytetracycline</th>
<th>Tylosin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yolk</td>
<td>White</td>
</tr>
<tr>
<td>1</td>
<td>601.9 ± 312</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>2</td>
<td>406.2 ± 283</td>
<td>109.9 ± 13.5</td>
</tr>
<tr>
<td>3</td>
<td>386.1 ± 183.8</td>
<td>95.2 ± 63.8</td>
</tr>
<tr>
<td>5</td>
<td>226.3 ± 175.5</td>
<td>39.9 ± 30.9</td>
</tr>
<tr>
<td>6</td>
<td>131.9 ± 41.8</td>
<td>38.4 ± 13.3</td>
</tr>
<tr>
<td>7</td>
<td>128.2 ± 95.7</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>78.5 ± 32.2</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>58.1 ± 18.5</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>53.1 ± 8.5</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>42.2 ± 24.4</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>37 ± 14.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) ND, nondetected.  
\(^b\) NE, nonestimated.
antimicrobial agents deplete more slowly in this egg component. Previous studies also consider the egg yolk as the main compartment for quantifying drug residues in eggs (19, 29, 30, 32), while some other authors have used the whole egg as target tissue (4, 8, 31). However, the use of both components as one matrix can be considered as a drug dilution factor. In this study, yolk and white were analyzed separately to evaluate differences in the concentration of the selected antimicrobials in both egg compartments.

The higher concentration reached by oxytetracycline in egg yolk in relation to tylosin concentrations, can be attributed to different factors. The pharmacokinetic characteristics of both antimicrobials differ such that absorption and bioavailability of oxytetracycline is higher when given orally. The low binding of oxytetracycline to plasmatic proteins and its moderate apparent volume of distribution, contrasts with tylosin characteristics. Other factors that should be considered are the physicochemical properties of the drugs: the molecular weight in oxytetracycline is almost half of tylosin’s molecular weight, facilitating passing through the tissue membranes of the former. In addition, the liposolubility of oxytetracycline favors this drug reaching the peripheral tissues with high lipid content, in comparison with tylosin, a hydrophilic molecule (1, 3, 23, 26).

Another factor that determines the antimicrobial accumulation in the egg components is the egg formation physiology. Hekman and Schefferlie (24) conducted experimental simulations in laying hens to provide a mathematical model based on the main parameters that could affect the occurrence of antimicrobial residues in eggs. These authors considered egg formation physiology, the egg physicochemical characteristics (lipid solubility and \( pK_a \)), and egg pharmacokinetic characteristics (plasma concentrations, half-life, and plasma protein binding), concluding that lipid-solubility and the pharmacokinetic parameters are responsible for the antimicrobial agent’s depletion, regardless of the follicle physiological formation stage. Other studies have pointed out that lipid solubility of antimicrobial agents and the egg formation physiology are the parameters that affect disposal and depletion of these drugs (13, 25). In the present study, this variable proves to be relevant in determining if a drug accumulates for a longer time in the egg yolk than in the white, because both drugs were distributed in the egg yolk via the blood at different concentrations; only oxytetracycline reached steady concentrations above the MRL for 5 days.

The obtained results suggest that the higher transfer of oxytetracycline into egg compartments compared with tylosin was influenced by the physicochemical properties. These properties influence the distribution of the studied antimicrobials, favoring a higher drug deposition in the egg yolk than in the white. These results concur with Vandenberge et al. (32) and Zurhelle et al. (34), who reported that tylosin and oxytetracycline reached higher concentrations in egg yolk than in white, respectively. Meanwhile, Alaboudi et al. (2) found higher concentrations of chlortetracycline in egg white than in yolk, even though they state that tetracycline residues are expected to concentrate in the egg yolk. They attribute this difference to unspecified multifactorial causes.

For tylosin, even though it presented the same distribution as oxytetracycline for both egg components, its pharmacokinetics properties and its molecular weight resulted in the lower transfer concentrations of this compound to the analyzed eggs. Other researchers have also detected low transfer ratios of tylosin to the eggs (20, 23, 32), while oxytetracycline concentrations were over the MRL for the first 5 days posttreatment.

The present study confirmed the presence of antimicrobial residues in eggs after oral therapy with oxytetracycline and tylosin in laying hens and established the WDTs for both studied drugs in egg white and yolk. Additionally, this is the first study in which concentrations of both drugs were analyzed in egg yolk and white separately, using liquid chromatography coupled to a diode array detector (22). This information points out the rising need of surveillance programs to monitor drug residues in different edible animal products in developing economies.

REFERENCES


