Serum and tissue concentrations of doxycycline in broilers after the sub-cutaneous injection of a long-acting formulation

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Abstract

1. The antibacterial agent doxycycline hyclate (Dox) is usually administered to broilers in drinking water or as a feed supplement. Parenteral injection is not the usual route for administration, so a long-acting formulation (Dox-LA) was tested to evaluate if serum concentrations can achieve the pharmacokinetic/pharmacodynamic (PK/PD) ratios regarded as adequate for the drug.

2. A poloxamer-based matrix was used to provide Dox-LA. Serum and tissue concentrations of Dox vs time were determined in two day-old broilers after subcutaneous (SC) injection of Dox-LA or oral administration of a single bolus of aqueous Dox (Dox-PO), at a dose of 20 mg/kg. Weight gain, feed conversion rate, haematological variables, aspartate aminotransferase and alanine aminotransferase activities, blood urea and creatinine were determined and compared for Dox-LA with Dox-PO and non-medicated controls.

3. Dox-LA had a high relative bioavailability (1200%). Maximum serum concentrations were not statistically different (5.1 ± 1.1 µg/ml for Dox-LA and 6.1 ± 1.4 µg/ml for Dox-PO), but half-life of Dox-LA was much greater than the value obtained for Dox-PO (73.0 ± 0.9 h and 2.0 ± 0.02 h, respectively). Tissue concentrations were higher, and stayed higher for longer periods in the Dox-LA group.

4. In conclusion, considering the minimum effective serum concentration against Mycoplasma spp is 0.5 µg/ml, a dose-interval of 180 h can be achieved with Dox-LA, but only for 24 h after Dox-PO. Better PK/PD ratios for Dox-LA should result in improved clinical outcomes compared with Dox-PO.

INTRODUCTION

Parenteral administration of drugs to newly-hatched broilers is not a common practice, this route being reserved for vaccines only (Vermeulen et al., 2002). When necessary, metaphylactic and therapeutic doses of antibacterial drugs are administered to broilers with the drinking water or as in-feed medication during the first days after hatching; for example, to control Mycoplasma spp and associated infections (Ismail and El-Kattan, 2004; Stipkovits et al., 2004; Pérez et al., 2006). Among such antibacterial drugs, doxycycline (α-6-deoxy-5-hydroxytetracycline) has gained a reputation as a valuable therapeutic agent. Doxycycline is a tetracycline derivative with broad spectrum activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria: Spirochaete, Mycoplasma, Chlamydia and Rickettsia species (Shaw and Rubin, 1986; Dorrestein et al., 1990; Goren et al., 1998; Riviere and Spoo, 2003). Doxycycline has some advantages over older tetracycline derivatives including higher lipid solubility, better bioavailability and tissue distribution, longer elimination half-life, and lower affinity for calcium ions (Aronson, 1980).

The pharmacokinetics of doxycyclin after oral administration have been studied in healthy chickens (Anadon et al., 1994; Laczay et al., 2001)
and in \textit{Mycoplasma gallisepticum}-affected broilers (Ismail and El-Kattan, 2004; Pérez et al., 2006). Bioavailability has been determined as approximately 65–70% but there is no parenteral formulation available due to causing severe tissue irritation. A possible exception is an experimental injectable long-acting doxycycline-hyclate (Dox-LA) preparation that causes minimum irritation and exhibits high bioavailability values (>600%) in goats (Vargas et al., 2008), calves (Vargas-Estrada et al., 2008a) and rats (Vargas-Estrada et al., 2008b). Considering the above, it was considered useful to evaluate and compare the pharmacokinetic parameters of this preparation with two-day-old broilers compared with those obtained after oral administration of an aqueous doxycycline solution (Dox-PO).

\section*{MATERIALS AND METHODS}

\subsection*{Experimental animals}

The study was approved by the Institutional Committee of Research, Care and Use of Experimental Animals of the Universidad Nacional Autonoma de Mexico, according to the Mexican Official Regulation NOM-062-ZOO-1999. The experiment was carried out in isolation units at the Universidad Nacional Autonoma de México in Mexico City, with a total of 1,400 two-day-old broiler chickens having a mean (±SD) weight of 48±2.4 g as determined by weighing 10% of the population. Upon arrival, broilers were allowed to settle and water was made available \textit{ad libitum} through nipple-type dispensers at 20 birds per cup (Marks, 1981; Quintana, 1988). House temperature was maintained at 30±1.5°C using gas heaters and a thermostat.

\subsection*{Long-acting Dox-LA preparation}

A subcutaneous Dox-LA preparation (100 mg/ml) of doxycycline hyclate (PARFARM Pharmaceuticals, Mexico City, Mexico) was produced under sterile conditions. Inclusion complexes of doxycyclin 10% (w/v) with β-cyclodextrin (1:0:1 m) (Cerestar Pharmaceutical Excipients, Hammond IN, USA) were first formed by the kneading method which can be described as follows: β-cyclodextrin (0-1 m) and distilled water were mixed together in a mortar to produce a homogeneous paste. Doxycycline (1 m) was added slowly. The mixture was ground for 30 min and an appropriate quantity of water added to produce a paste-like consistency. This was dried at 40–50°C for 24 h. The dried complex was pulverised into a fine powder (Bekers et al., 1991). The resulting powder was diluted with a solution of 15% propylene-glycol – 10% ethyl alcohol in water. This mixture was included in a reverse gel copolymer polyoxypropyl-polyoxyethylene (poloxamer) (BASF, Mexico City, Mexico), adjusting pH to 7.0 with a phosphate buffer solution with constant stirring at 4°C to produce a final concentration of 10% Dox in 15% poloxamer. The preparation was completed when a micro-emulsion was formed and the mixture clarified. Quantities (10 ml) were dispensed into vials, stored at 4°C and utilised during the following week.

\subsection*{Experimental design}

A total of 5 groups were formed as follows. Group 1 containing 400 broilers was dosed with the experimental preparation (Dox-LA) at approximately 1 mg/broiler (~20 mg/kg) in a total volume of 200 µl using a precision syringe (syringe for oily vaccine, Broiler ND K®, CEVAC; Barcelona, Spain) for pharmacokinetic determination. Group 2 (200 broilers) received a similar Dox-LA dose and these broilers were used to investigate any side effects such as tissue damage. Production variables were determined during the following 6-week period. In both these groups, birds were observed for up to 20 min after injection to detect any changes in behaviour. Groups 3 (400 broilers) and 4 (200 broilers) were given oral doxycyclin (Dox PO) to compare the pharmacokinetics and production variables, respectively. These two groups were treated with a single oral dose of a freshly diluted 0-5% aqueous solution of doxycycline hyclate by means of rigid tubing into the proventriculus in a volume of 200 µl. Group 5 was the untreated control group (CG with 200 broilers) and dosed orally with 200 µl of saline solution.

Sampling times were selected to provide the least amount of error in the estimate of the pharmacokinetic parameters. To analyse concentrations of doxycycline in tissues, 15 broilers from each group were killed, as specified by Mexican regulations (NOM-009-ZOO-1994), 6 h after dosing and then daily for 6 d. The small intestine, large intestine plus caecum-rectum, liver, lungs and yolk sac from each animal were dissected and stored in Eppendorf tubes at −20°C until analysed. Because of the small amount of small intestine and yolk sac collected from each broiler, three separate samples, each made up of tissue from 5 broilers, were needed to obtain sufficient material for determination of doxycycline concentration. Yolk sac was only determined for 5 d (7 day-old broilers) until this tissue was no longer detectable. Tissue reactions at the site of injection were investigated in all broilers from the Dox-LA-treated groups during an 8 d follow-up period. For groups Dox-LA (Group 1) and Dox-PO (Group 3), 5 broilers...
were sampled for blood by direct jugular puncture using a one ml syringe and 30 gauge needle at the following times: before treatment and at 1, 2, 4, 6, 8, 12, 24, 26, 28, 30, 32, 36, 48, 50, 52, 54, 56, 60, 72, 74, 76, 78, 80, 84, 96, 98, 100, 102, 104, 108, 120, 122, 124, 126, 128, 132, 144, 146, 148, 150, 152, 156, 168, 170, 172, 174, 176, 180 and 196 h. In Group 2, Group 3 and Group 5, blood samples were obtained from 10 broilers randomly chosen before the start of the experiment, and at weekly intervals for 6 weeks for haematological analysis (cell volume, red blood cell counts, total white blood cell count and haemoglobin concentration). Aspartate aminotransferase and alanine aminotransferase enzyme activities were determined using an autoanalyser (CELLY 70 Autoanalyser, Chronolab SA de CV, Mexico City). Finally, blood urea nitrogen and creatinine were determined as described by Boisness and Taussky (1985). Production variables (weekly weight gain, feed conversion ratio and cumulative mortality) were assessed in these three groups.

Within the next three weeks from the end of the experiment, samples were thawed and doxycycline concentrations in serum and tissue samples determined by the quantitative/qualitative agar plate diffusion method (Bennet et al., 1966) using Müller Hinton medium (Difco Laboratories, Detroit, MI, USA) and Bacillus cereus var. mycoides (ATCC, 11778) as test organism. This method measured concentration in terms of the in vitro antibacterial activity. The chosen bacterium was seeded at an approximate inoculum of 5 x 10^5 CFU/ml in Mueller-Hinton Agar (Bioxon/Proveedor Cientifico, Mexico City, Mexico) and using chicken serum as diluent. No baseline activity with blank serum was detected. Percentage recovery achieved with this technique was 92.6%, with intra- and inter-assay coefficients of variation of 5% and 6%, respectively.

A computerised curve stripping program, PKAnalyst (MicroMath®, Saint Louis, Missouri, USA), was used to fit and analyse the concentration-versus-time patterns for each group. Models of best fit (r ≥ 0.99) were chosen after analysis by use of residual sum of squares and the minimal Akaike’s information criterion (Welling, 1997). Best fit for the SC route was obtained by use of model 3 whose general formula is:

$$\text{Concentration (Time) } = \frac{\text{Dose}}{\text{Volume}(K_{ab} - K_d)} \cdot K_{ab} \cdot e^{-K_e \text{Time}} - e^{-K_{ab} \text{Time}}$$

Variables obtained were: $AUC_t = \text{area under the curve;}$ $AUMC_{0-\infty} = \text{area under the first moment curve from 0 to } \infty$ with extrapolation of the terminal phase; MRT = mean residence time; $\beta = \text{rate constant for the elimination phase;}$ $T_{1/2,\beta} = \text{elimination half life;}$ $C_{\max} = \text{serum peak concentration;}$ $T_{\max} = \text{peak time.}$

For Dox-PO, the following general formula was adopted:

$$\text{Concentration (Time) } = A e^{-\alpha \text{Time}} + B e^{-\beta \text{Time}} + C e^{-\lambda \text{Time}}$$

Variables obtained were as above. Relative bioavailability was calculated as follows:

Relative bioavailability

$$AUC_{\text{Dox–PO}} / AUC_{\text{Dox–LA}} \times 100$$

Data are presented as mean ± standard deviation for three sets of observations for each parameter; and for statistical comparisons of $C_{\max}, T_{\max}, AUC_t, \text{MRT and } T_{1/2,\beta}$ among groups the ANOVA was followed by a Bonferroni-test.

**RESULTS**

Broilers did not manifest signs of pain or discomfort after injection of the Dox-LA formulation, and no inflammatory response was evident at the injection site. A small painless swelling was detectable in some broilers. These swellings had an initial size of approximately 2-4 mm in diameter and gradually disappeared by d 7 post injection.

Haematological, as well as blood urea and creatinine and liver enzyme activities remained within the accepted limits for the species and a distinguishable pattern or change between groups was not detected (data not shown). Table 1 shows weight gain, feed conversion ratio and
cumulative mortality in groups 2, 4 and 5. Mean serum concentration-versus-time patterns of doxycycline for group 1 (Dox-LA) and group 3 (Dox-PO) are shown in Figures 1 and 2 and the determined pharmacokinetic variables are summarised in Table 2. Comparisons for AUC, T1/2, and T95% showed that all these variables were significantly greater in the Dox-LA treated group than in the Dox-PO group (P < 0.01). The difference between mean elimination half-lives of 73 ± 0.9 h for Dox-LA and 2 ± 0.2 h for Dox-PO was highly significant (P < 0.001). In contrast, Cmax was greater in the Dox-PO group (6.1 µg/ml in Dox-PO vs. 5.1 µg/ml in Dox-LA) (P < 0.05).

A flip-flop pharmacokinetics of this preparation is postulated applying Boxenbaum (1998) and Laczay et al. (2001) criteria with the following equation:

\[ \text{Rate of absorption} = V_z(KC + (\Delta C/\Delta t)) \]

where \( V_z \) is the terminal exponential volume of distribution; \( K \) is the terminal disposition rate constant once drug absorption is complete (best determined from IV dosing); \( C \) is the plasma concentration at time \( t \); and \( \Delta C \) is the change in plasma concentration over the time interval \( \Delta t \).

For Dox-LA plasma concentration-time data at 24 and 48 h, \( \Delta C/\Delta t = 0.03 \) µg/ml/h. At the midpoint of this time period (36 h), \( (K)(C) = 4.1 \) µg/ml/h. Since \( KC \gg \Delta C/\Delta t \), and rate of absorption \( \approx \) rate of elimination, a “flip-flop” condition exists and the Dox-LA here described can be regarded as a true long-acting one.

**DISCUSSION**

The agar diffusion technique used here to determine serum and tissue concentrations of

![Figure 1](image-url)

**Figure 1.** Mean and SD serum concentrations of doxycycline after the subcutaneous injection of a single dose of 20 mg/kg of a doxycycline-LA experimental preparation (Dox-LA), and after administering the same dose orally with a semi-rigid tube (Dox-PO).
Figure 2. Mean ± 1 SD concentrations of doxycycline in lungs, small intestine, vitellin sac, large intestine plus caecum, and liver after the subcutaneous injection of a single dose of 20 mg/kg of a doxycycline-LA experimental preparation (Dox-LA, n = 15) in a total volume of 200 μl on the second day after hatching; or after administering an aqueous solution of the drug by directly depositing it into the crop with a semi-rigid tube (Dox-PO, n = 15) using the same dose and volume.

Table 2. Mean ± SD pharmacokinetic parameters in broilers after a single dose of doxycycline (20 mg/kg) administered either by subcutaneous injection (Dox-LA) or orally (Dox-PO).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dox-LA</th>
<th>Dox-PO</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_i (μg h/ml)</td>
<td>611.5 ± 21.3</td>
<td>48.6 ± 6.6</td>
</tr>
<tr>
<td>AUMC_Co~∞ (μg h/ml)</td>
<td>66786 ± 956.2</td>
<td>282.1 ± 10.5</td>
</tr>
<tr>
<td>T_1/2β (h)</td>
<td>109.2 ± 7.3</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>T_1/2ab (h)</td>
<td>73.0 ± 0.9</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>K_{ab} (h)</td>
<td>2.7 ± 0.1¹</td>
<td>2.0 ± 0.1²</td>
</tr>
<tr>
<td>K_e (h)</td>
<td>0.19 ± 0.08</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>C_{max} (μg/ml)</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>51 ± 1.1</td>
<td>61 ± 1.4</td>
</tr>
<tr>
<td>V_d/AUC (l/kg)</td>
<td>13.4 ± 5.2</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>Cl (ml/kg/h)</td>
<td>28.61 ± 5.25¹</td>
<td>4.11 ± 0.71</td>
</tr>
<tr>
<td>Fr (%)</td>
<td>0.001</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td></td>
</tr>
</tbody>
</table>

¹ Calculated for flip-flop pharmacokinetics (Concordet, 2010).

Doxycycline is a dependable method, allowing pharmacokinetic data to be extrapolated to antibacterial activity with certainty (Santos et al., 1996; Vargas et al., 2008; Vargas-Estrada et al., 2008a).

The pharmacokinetics of doxycycline have been reported in chickens following oral, intravenous (IV) and intramuscular (IM) administration of the drug (Anadón et al., 1994; Laczay et al., 2001; Atef et al., 2002; Ismail and El-Kattan, 2004; El-Gendi et al., 2010). The elimination half-life of doxycycline following oral administration varies with age between 10 and 12 h (Pashov and Kamelov, 1994; Hantash et al., 2008). However, these values are notably different from the $T_{1/2\beta}$ values of 3.64 to 4.75 h reported by Anadón et al. (1994), Atef et al. (2002) and El-Gendi et al. (2010). In the present study a $T_{1/2\beta}$ of 2.0 ± 0.02 was determined and this may be in part explained by the use of an oral bolus dose. Elimination half-life after IM and SC administration of a large dose of 100 mg/kg were reported...
to be 86 and 63 h, respectively (Greth et al., 1993). In contrast, Atef et al. (2002) report a half-life of only 2.5 h after the IM administration of 15 mg/kg. In the present study, \( T_{\beta} \) in the group receiving Dox-LA was 73 h. These differences may be explained by the dose (Greth et al., 1993) causing an inflammatory reaction and a depot-like effect, whereas a real depot effect is likely to have been obtained in this study without an inflammatory reaction.

Maximum serum concentrations in the Dox-LA and Dox-PO groups (5–6 µg/ml) were higher than values obtained after dosing fasted or fed broilers with this drug (3–0.7 µg/ml to 4.47 µg/ml, respectively; Laczay et al., 2001), but they were similar to those reported by Atef et al. (2002) after IM administration of 15 mg/kg (6.33 µg/ml) and Hantash et al. (2008) after oral administration of 20 mg/kg.

Predictably, for a highly lipid soluble drug such as doxycycline, a high value of the apparent volume of distribution \( AUC \) was achieved with Dox-LA, with a very small total body clearance. The apparent \( V_{d\text{app}} \) value derived from Dox-PO is similar to the one reported by Hantash et al. (2008) and almost double that reported by Atef et al. (2002). However, no studies are available on the pharmacokinetics following SC injection of a long acting preparation.

Relative Dox bioavailability for the Dox-LA group was 1200% as compared with the Dox-PO group. Absolute bioavailability in other studies was never greater than 70% when comparing the oral with IV route (Anadón et al., 1994). Although these values of \( F \) cannot be directly compared, differences appear substantial. This is not uncommon for formulations with prolonged absorption, exhibiting flip-flop kinetics (Boxenbaum, 1998; Toutain and Bousquet-Mélou, 2004). A recycling phenomenon due to the noticeably high lipid solubility of Dox may have contributed to these differences (Aronson, 1980; Chopra et al., 1981).

The rationale behind the design of the Dox-LA preparation was to use the poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) block copolymer (poloxamer) as delivery vehicle-matrix, because it improves solubility, reduces hydrolytic degradation, achieves controlled release and often results in improved bioavailability (Tarr and Yalkowsky, 1987; Kabanov et al., 1992). The poloxamer exhibits low viscosity at room temperature (28–32°C) and becomes a gel at body temperature (37–40°C) (Schmolka, 1991), thus allowing the long-acting effect observed. Additionally, reduced irritation is postulated to be due to \( \beta\)-cyclodextrin by reducing the local concentration of free drug below the irritancy threshold (Szejtli, 1985; Yoshida et al., 1990; Uekama et al., 1998).

Additionally, a priming effect is obtained (Yoshida et al., 1990; Uekama et al., 1998). Because injection of Dox-LA failed to induce significant changes in haematological variables, or a reduction in production values, lack of toxicity of this drug preparation is envisaged.

It has been postulated that maximum efficacy in a clinical setting with Dox is achieved when serum concentrations of the drug are barely at or above the MIC level for the pathogen in question, for as long as possible within the dosing interval (Craig, 1998; Prescott et al., 2000). Values of MIC that can be adopted in this experiment can be categorised as susceptible for Mycoplasma gallisepticum (0.2 µg/ml: Takahashi and Yoshida, 1989; Stipkovits et al., 2004) and less susceptible for Escherichia coli (1–4 µg/ml: Cunha et al., 1982). Additionally, Notari (1987) suggested a MTC from 0.5 to 1 µg/ml for this antibacterial drug and a similar value has been advanced for broilers (Hantash et al., 2008). Considering the above, a MTC range for susceptible bacteria can be set from 0.5 µg/ml to 1.5 µg/ml. Hence, the length of time in which MTC can be achieved with Dox-PO varies from 10 h to 24 h, and for Dox-LA this interval is extended from 150 to 180 h. If lung concentrations of doxycycline are considered as key values to design a dosing scheme, then SC administration of Dox-LA is considerably superior as compared with administering doxycycline through the drinking water. Concentrations in lung higher than 0.4 µg/ml are extended until d 6 in the Dox-LA group with a single dose of 20 mg/kg, injected SC. This is consistent with the unusually large \( V_{d\text{app}} \) obtained in this group.

The PK/PD index, best accepted as a predictor of therapeutic efficacy for tetracyclines in humans, is the ratio \( AUC \)/MIC (Baños and Ferré, 2002). Whereas \( AUC_{ss} \) was not evaluated in this study, if a pathogen is susceptible at 1.5 µg/ml, the \( AUC_{\text{Dox-LA}}/\text{MIC} \) ratio achieves a value of 407. In contrast, the \( AUC_{\text{Dox-PO}}/\text{MIC} \) ratio is only 32. Differences are considerable and clinical efficacy should be higher. Additionally, Dox-LA provides reasonably steady serum concentrations for 5 d, while oral administration of the drug over such a period will vary according to water consumption with daily peaks and troughs.

Doxycycline is effective against respiratory pathogens in avian medicine, as well as in other domestic species, because it penetrates well into respiratory tissues (Anadón et al., 1994; Atef et al., 2002; Ismail and El-Kattan, 2004). By injecting Dox-LA, this feature seems to be boosted, and is longer-lasting compared with Dox-PO. This is likely to be beneficial when treating susceptible pathogens such as Mycoplasma spp., E. coli and others. Additionally, the gastrointestinal system
is one of the main routes for elimination of doxycycline, which is therefore effective against colibacillosis and other enterobacterial infections, as the same reasoning applies.

In conclusion, Dox-LA is a preparation that optimises PK/PD congruency in broilers and provides at least 4 d of medication, making it more cost effective and perhaps more ecologically sound. Nevertheless, clinical trials and drug-residue studies are needed to assess if this preparation can be regarded as potentially useful at a production level. Further assessment of the safety of this preparation in broilers is required.

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