Received: 10 January 2020 | Revised: 27 February 2020 | Accepted: 9 March 2020

DOI: 10.1111/jvp.12859

PHARMACOKINETIC REPORT

JOURNAL OF Veterinary Pharmacology and Therapeutics WILEY

Oral doxycycline pharmacokinetics: Lambs in comparison with sheep

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Abstract

The pharmacokinetics of doxycycline was investigated in lactating sheep and lambs after oral administration at a dose of 10 mg/kg. Concentrations in plasma and milk were assayed with HPLC-PDA analysis. Doxycycline penetrates into the milk, and levels (0.38 ± 0.21 μg/ml) were found 0.5 hr after the treatment. The results suggest that the lambs can be exposed to doxycycline by suckling milk from their treated mothers. Population pharmacokinetic analysis showed a positive relationship between age, which reflects the stage of development of rumen function, and clearance. Possible explanations for the observed differences include the undeveloped rumen in lambs, the differences in the feed and liver function as evidenced by the blood biochemical parameters aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which were significantly lower in lambs (62.67 ± 27.83 U/L and 8.50 ± 6.80 U/L) than in sheep (114.33 ± 20.77 U/L and 18.00 ± 3.16 U/L).

KEYWORDS

age differences, doxycycline, pharmacokinetics, small ruminants

1 | INTRODUCTION

Current good veterinary practice is primarily targeted toward maintaining animal health and minimizing the use of antimicrobials. If antimicrobials are used for the treatment of infectious diseases, it must be justified and grounded in sound scientific principles. Knowledge of the factors that influence antimicrobial pharmacokinetics is needed to optimize dosage regimens.

Tetracyclines are one of the antimicrobial groups authorized for use in veterinary medicine. Because of their wide spectrum of activity and limited adverse effects, they are widely used to control various bacterial pathogens. In addition, they are effective for the treatment of protozoal infections, which is of increasing importance due to their rising prevalence in endemic regions (Yang et al., 2016).

Doxycycline is a tetracycline that has numerous advantages over the other members of the group (del Castillo, 2013). It shows broader spectrum of activity, higher oral bioavailability, and distributes intracellularly and to sites in the body that are protected by diffusion barriers (e.g., brain). Doxycycline has a longer elimination half-life and a lower affinity for Ca^{2+}. Furthermore, it forms a stable solution in water, which makes it suitable for administration through the drinking water, a convenient and relatively inexpensive route of administration (del Castillo, 2013).

Because of these advantages, it is often used in veterinary practice (De Briyne, Atkinson, Borriello, & Pokludová, 2014; Granados-Chinchilla & Rodríguez, 2017). The pharmacokinetics of doxycycline has been reported in many mammalian species such as horses, pigs, dogs, and cats (Bousquet, Nouws, Terlouw, & Kleyne, 1998; Bousquet, Richard, Fourtillan, Girault, & Istin, 2003; Hartmann, Krebber, Daube, & Hartmann, 2008; Zozaya, Gutierrez, Bernad, & Sumano, 2013). Limited data are available in small ruminants (Abd El-Aty, Goudah, & Zhou, 2004; Castro et al., 2009) and in large ruminants (Riond, Tyczkowska, & Riviere, 1989). Some investigations have revealed differences in the pharmacokinetics of doxycycline between ruminants and monogastric animals on the one hand and between young calves and mature ruminants on the other hand (Riond et al., 1989). Castro et al. (2009) have found that the rate of absorption after oral administration of doxycycline in sheep was

slower than in pigs and preruminant calves. After intravenous doxycycline administration, the values of clearance in calves with immature rumen function were higher than in calves with mature function (Riond et al., 1989).

Published data about the differences in doxycycline pharmacokinetics after oral administration between young and mature small ruminants were not found in the available literature. These variations are particularly important in ruminants because of the anatomical and functional features of their gastrointestinal tract which are highly depend on the age (de Backer & Bogaert, 1983). Therefore, better understanding of the influence of age, development of the digestive system, and diet on the pharmacokinetics of doxycycline in small ruminants is needed.

An additional source of differences can be lactation in ruminants. It is known that doxycycline crosses the blood–milk barrier and penetrates into the milk (Šopík, Vydrová, Zálešáková, & Buňka, 2016). Suckling animals can therefore be exposed to the drug through milk.

Taking into account the above information and the growing importance of doxycycline in the treatment of respiratory and gastrointestinal infections in sheep, as well as anaplasmosis, knowledge of age-dependent doxycycline pharmacokinetics is needed. Therefore, the aim of the current study was to investigate the differences between the pharmacokinetics of doxycycline in sheep and lambs after oral administration and to determine the impact of the age on the absorption and disposition of this antimicrobial drug. It was also intended to obtain additional information about resulting concentrations in the milk of lactating sheep. Based on these results, dosage regimens may be adjusted to improve treatment outcomes for bacterial and protozoal diseases in sheep.

2 | MATERIAL AND METHODS

2.1 | Animals

Six adult female sheep and six lambs of their offspring aged 30 days were used in this study. They were local Bulgarian breed (Karnobat sheep). The mean bodyweight of the adults was 50.80 ± 4.67 kg and of the lambs was 14.48 ± 1.63 kg. Animals were determined to be clinically healthy by physical examination. The sheep were not used for milk production for human consumption. The trial was conducted in the holding where they were farmed and no acclimatization period was required.

Sheep were on a diet of hay and feed concentrate for small ruminants (HL-Top Mix, Sliven, Bulgaria). Lambs were still sucking but also had free access to alfalfa hay. Water was available ad libitum.

The experiment was conducted according to the requirements of Bulgarian legislation (Ordinance No 20/1.11.2012 on the minimum requirements for protection and welfare of experimental animals and requirements for use, rearing and/or their delivery).

2.2 | Study design

Three days prior to treatment, blood samples were collected from all animals for determination of blood biochemical parameters. Total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were evaluated. Blood samples were collected with collection test tubes (2.5 ml Lithium heparin, FL Medical, Italy). The biochemical parameters were determined on Biochemistry analyzer BS-120 Mindray in the Clinical Laboratory, Laboratory and diagnostic center, Trakia University. Milk and blood samples from untreated animals were collected, and they were used for preparation of standard curve for HPLC analysis.

On the day of the treatment, the sheep (n = 6) were separated from their lambs (n = 6) until the last sampling point. Both of them were fed before the experiment. Doxycycline was administered as a single oral dose of a 1% solution at 10 mg/kg to both the sheep and lambs. For that purpose, a commercial formulation (HydroDoxx 500 mg/g Oral Powder, Huvepharma) was used. It was dissolved in water ex tempore. The solution was administered orally via individual syringes. Blood samples (1.8 ml) were withdrawn from lambs and sheep from one of the jugular veins at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 14 and from sheep at 24 hr after drug administration with vacutainers (Cat No 4.18.1.3., LT Burnik, LTD, Slovenia). The blood sampling in younger animals was not possible after 14 hr due to ethical reasons and welfare of the animals. The lambs could not be separated from their dams for longer, and suckling would additionally expose them to doxycycline. Milk samples were collected at 0.5, 14, and 24 hr after the start of the treatment. The milk samples at 0.5 hr were only between 2 and 7 ml because the lambs had been allowed to suckle before treatment and there was little milk left in the udder. The sheep were not fully milked at 14 hr, but the milk was sucked from the lambs immediately after sampling. The amount of the milk that was possible to be collected at last sampling point was not more 10 ml per animal. Plasma was immediately separated by centrifugation and stored at − 20°C until analysis. Milk samples were stored at the same conditions.

2.3 | HPLC analysis of doxycycline concentrations

Plasma doxycycline concentrations were analyzed by HPLC with PDA detection using a method described by Laczay, Semjén, Lehel, and Nagy (2001) with minor modifications. Plasma samples (150 µl) were spiked with 15 µl internal standard (IS, oxytetracycline 11 µg/ml) and 19.5 µl trifluoroacetic acid (TFA). They were vortexed for 1 min and centrifuged for 10 min at 10,800 g at 22°C. The supernatant was placed in HPLC vials, and 20 µl of each sample was injected into the HPLC system. Milk samples (500 µl) were mixed with 50 µl IS and 65 µl TFA. After vortexing for 1 min and centrifugation for 10 min at 10,800 g at 22°C, the supernatant was transferred to another tube. These samples were centrifuged again for 5 min at 10,800 g at 22°C and then filtered through standard filter paper (pore size 10–20 µm). The filtrate was placed

in a vial, and 20 µl of each sample was injected into the HPLC system. It comprised a Hypersil Gold column (5 µM, 15 × 4.6 mm), a Surveyor LC Pump Plus, a PDA detector Surveyor, and a Surveyor Autosampler Plus (Thermo Fisher Scientific Inc.). The column was used at room temperature. The mobile phase consisted of acetonitrile, methanol, 0.02 M oxalic acid, and 0.02 M $Na_2H_2EDTA \times 2H_2O$ (20:15:64:1, v/v/v/v). The flow rate was 1.0 ml/min. The detection wavelength was set at 345 nm. Under these conditions, the retention times were 2.7 min for oxytetracycline and 5.7 min for doxycycline in both plasma and milk. Peak area integrations were measured by the ChromQuest Chromatography Data System (Thermo Fisher Scientific Inc.).

The limit of detection and the limit of quantification of the used method for the ovine plasma were 0.09 and 0.26 µg/ml and for the ovine milk 0.06 and 0.18 µg/ml, respectively. The mean accuracy of the method and mean extraction recovery of doxycycline determined in standard solutions in plasma were 95.17% and 91.01%. The same parameters for standard solutions prepared in milk were 100.96% and 91.16%. The mean intra- and inter-assay precision (RSD %) for the first biological matrix (plasma) were 5.57 and 11.11, and 3.94 and 6.84 for the milk (Mileva, 2019).

2.4 | Pharmacokinetic analysis

Pharmacokinetic analysis of the observed concentrations was performed by using a noncompartmental method for extravascular administration (Phoenix® 64 Build 8.1.0.34 software, Certara®). Area under the curve (AUC) was calculated according to linear up log down method. Area under the concentration–time curves from 0 to infinity ($AUC_{0-\infty}$) was also computed, but the percentage of extrapolation was higher than 20 and the values of AUC based on measured concentrations (AUC_{0-t}) were also presented. These data were further used with caution. Elimination rate constant (k_{el}) was estimated as the slope of the linearized terminal portion of the concentration–time determined by linear regression (concentration values were log-transformed). T_{max} was the time of maximum observed concentrations (C_{max}). For the sheep data, the observed concentrations were weighted by the $1/y^2$ scheme. This was not necessary for the lamb data as the difference between peak and last concentrations was not as large.

Population pharmacokinetic analysis was performed using Monolix® (Lixoft). Standard measures of goodness of fit were used to determine whether a one- or a two-compartment model (both with first-order absorption and elimination) described the data better. The measures of goodness of fit included observed-versus-predicted and residual plots and the value of the Akaike's information criterion (AIC). Once the basic structural model was established, the different co-variates (age, bodyweight, adult or immature, ALT, and AST activity) were explored to determine whether some of the variability in the observed data could be explained in a co-variate model. The pharmacokinetic parameters were assumed to be log-normally distributed, whereas the residual error in the measured plasma concentrations was assumed to be normally distributed. A combined (proportional and linear) error model was assumed for the fit.

TABLE 1 Biochemical parameters in lactating sheep (n = 6) and 30-day-old lambs (n = 6)

Biochemical parameter (unit)	Sheep	Lambs
AST (U/L)	114.33 ± 20.77	62.67 ± 27.83*
ALT (U/L)	18.00 ± 3.16	8.50 ± 6.80*
AST/ALT ratio	6.39 ± 0.78	9.23 ± 4.79
LDH (U/L)	1626.00 ± 294.09	1629.50 ± 129.29
Total protein (g/L)	72.97 ± 4.67	63.78 ± 11.13
Albumin (g/L)	34.40 ± 1.90	34.77 ± 1.15

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

*Statistically significant differences at $p < .05$.

2.5 | Statistical analysis

For the biochemical parameters, a Kolmogorov–Smirnov was applied to test for normal distribution of the data, which were then presented as mean ± SD and compared using a t test. Pharmacokinetic parameters were log(Ln)-transformed and tested for normal distribution using Kolmogorov–Smirnov test after which the data were reported as geometric mean ± geometric standard deviation. The t test was applied to the log(Ln) transformed values to test for statistically significant differences between the pharmacokinetic parameters of sheep and lambs. All analyses were run using a commercially available software (STATISTICA for Windows 7.0, StatSoft, Inc).

3 | RESULTS

The results from biochemical tests are summarized in Table 1. Significantly lower values of ALT and AST were found in lambs in comparison with the sheep. The other measured parameters (total protein, albumin, and LDH) had similar values.

No signs of discomfort were seen in any animal after oral administration of the drug. Mean plasma concentrations of doxycycline as a function of time after single oral administration of 10 mg/kg are presented on Figure 1. No lag time was observed in both groups. The concentrations in the last samples, taken 24 hr after oral treatment of sheep, were below the LOQ 0.26 µg/ml of the HPLC-PDA method. Traces of the antibiotic could be observed in these samples without possibility to quantify the concentration present (<LOQ), and they were not included in the pharmacokinetic analysis.

The value of $AUMC_{0-t}$ was significantly higher in lambs than in sheep ($p < .05$, Table 2). The values of C_{max} and T_{max} in the lambs were numerically higher ($p > .05$) compared with the adult animals (Table 2).

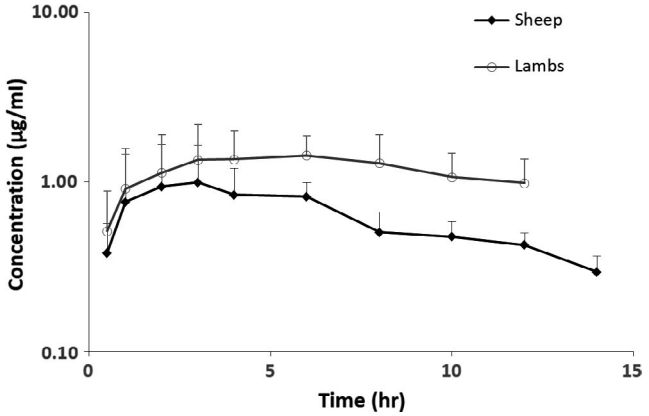


FIGURE 1 Semi-logarithmic plot of the mean ± *SD* doxycycline concentrations in plasma of sheep (n = 6) and lambs (n = 6), following a single oral administration of doxycycline at a dose rate of 10 mg/kg

TABLE 2 Pharmacokinetic parameters (Geometric mean ± geometric *SD*) obtained in sheep (n = 6) and lambs (n = 6) after oral administration of 10 mg/kg doxycycline

Parameter	Sheep	Lambs
Noncompartmental analysis		
k_{el} (hr^{-1})	0.126 ± 0.03	0.101 ± 0.04
T_{max} (hr)	3.46 ± 1.99	6.41 ± 2.69
C_{max} (µg/ml)	1.01 ± 0.65	1.55 ± 0.78
AUC_{0-t} (hr * µg/ml)	7.96 ± 2.81	12.64 ± 5.52
$AUC_{0-\infty}$ (hr * µg/ml)	10.52 ± 2.83	22.57 ± 9.26
% of extrapolation of AUC	21.78 ± 10.89	37.92 ± 20.76
$AUMC_{0-tl}$ (hr * hr * µg/ml)	50.08 ± 10.74	77.08 ± 29.80*
MRT (hr)	9.85 ± 2.69	12.73 ± 4.73

Abbreviations: $AUC_{0-\infty}$, area under the concentration–time curves from 0 to infinity ∞; AUC_{0-t}, area under the concentration–time curves on the basis of measured concentrations during the treatment; $AUMC_{0-t}$, area under the moment curve from the time of dosing to the last measurable concentration; C_{max}, maximum plasma or milk levels; k_{el}, elimination rate constant; MRT, mean residence time on the basis of the observed data; T_{max}, time of C_{max}.

*Statistically significant differences at $p < .05$.

The concentrations of doxycycline in the milk at 0.5 hr after the treatment were 0.38 ± 0.21 µg/ml. The values were similar to these in the plasma at the first sampling point. They were statistically higher in the milk (0.59 ± 0.29 µg/ml, $p < .05$) than in the plasma 14 hr after the treatment, just before sucking by the lambs. Doxycycline was found at measurable concentrations in the milk 24 hr after the treatment (0.29 ± 0.1 µg/ml) when the levels in the plasma could not be quantified.

Using the population approach, a one-compartment model with first-order absorption and elimination was judged to fit the data well without any systematic bias in the predictions of plasma concentrations (Figure 2a). The average apparent clearance (clearance confounded by an unknown bioavailability) was approximately double in the adult animals compared with the lambs (Figure 2b). Therefore, in the final model, a categorical variable classifying an individual as either adult (1) or immature (0) was included as a co-variate for apparent clearance in the final model (Equation 1, Table 3). This gave a significantly improved fit with the AIC value decreasing from 14.71 to 3.44.

$$\text{Log}\,(CL/F_i) = \text{Log}\,(CL/F_{pop}) + \beta_{CL/F\,adult}\,1_{adult=1} + \text{eta}_{CL/F,I} \qquad (1)$$

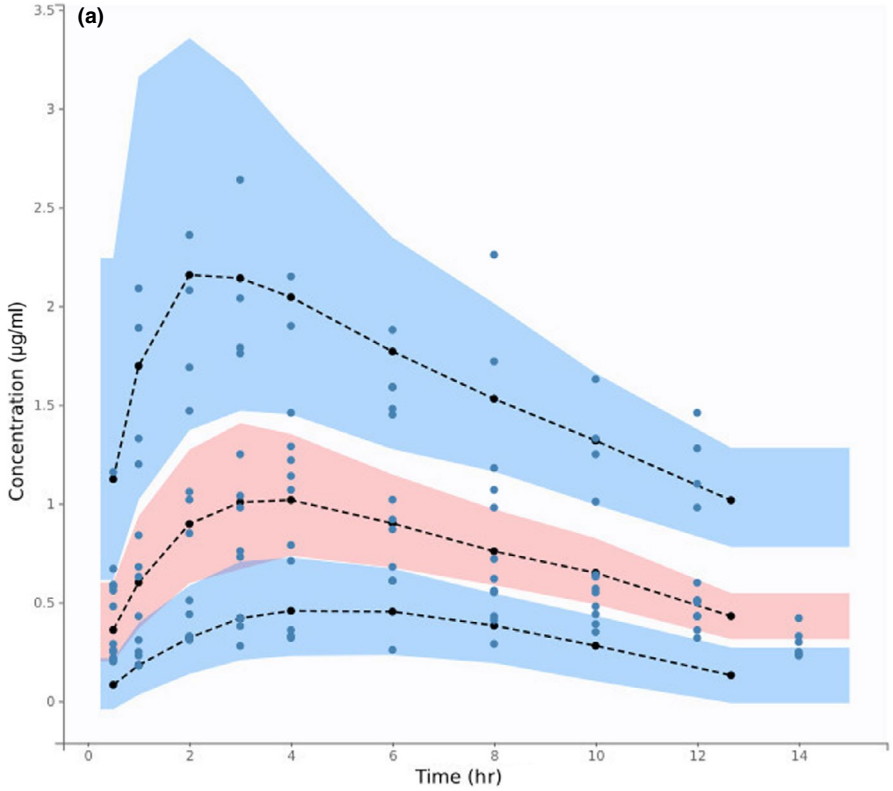
where CL, clearance, F, bioavailability, i, individual value, pop, typical value for the population, and β is the co-variate parameter adjusting the typical population value for CL for sheep because the co-variate "adult" is set to 1. (Lambs set to 0).

The continuous variables AST and ALT were considered as co-variates for the apparent volume of distribution, but they were not included in the final model as they did not improve the model significantly and increased the uncertainly of some of the parameter estimates.

4 | DISCUSSION

Over the last four decades, several studies of the pharmacokinetics of doxycycline in ruminants, including in lactating goats and cows, have been published (Abd El-Aty et al., 2004; Jha, Jayachandran, Singh, & Singh, 1989; Ziv & Sulman, 1974). The investigation of Castro et al. (2009) described its pharmacokinetics in adult sheep without any data about the lactation of the animals. According to our knowledge, there are no data in the available literature about the age-dependent differences in oral pharmacokinetics of doxycycline in lactating sheep and their offspring and the present investigation reveal some variations between both groups.

Previous investigations with adult sheep used two-compartmental model to characterize the pharmacokinetics of doxycycline after administration of 20 mg/kg oral dose (Castro et al., 2009). The cited study described late elimination phase, 24 hr after treatment and detectable concentrations until 72 hr after doxycycline administration. As a limitation of the present study can be discussed, the sensitivity of the HPLC-PDA method and low concentrations after 24 hr which could be detected as traces of doxycycline. The higher dose (2×) used in the study of Castro et al. (2009) with sheep explains the twofold higher values of C_{max}. The values of T_{max} are close to those reported by Castro et al. (2009). There are no previous data available describing doxycycline pharmacokinetics after oral administration in small ruminants with immature rumen function. Therefore, the results in lambs cannot be discussed in comparative way to the data from other studies in lambs or kids. Comparison between calves and lambs reveals species-specific differences in doxycycline pharmacokinetics. Calves, with immature rumen function, treated with 10 mg/kg orally show twofold lower values of C_{max} and AUC and shorter T_{max} than in lambs (Meijer, Ceyssens, Greve, & Bruijn, 1993).



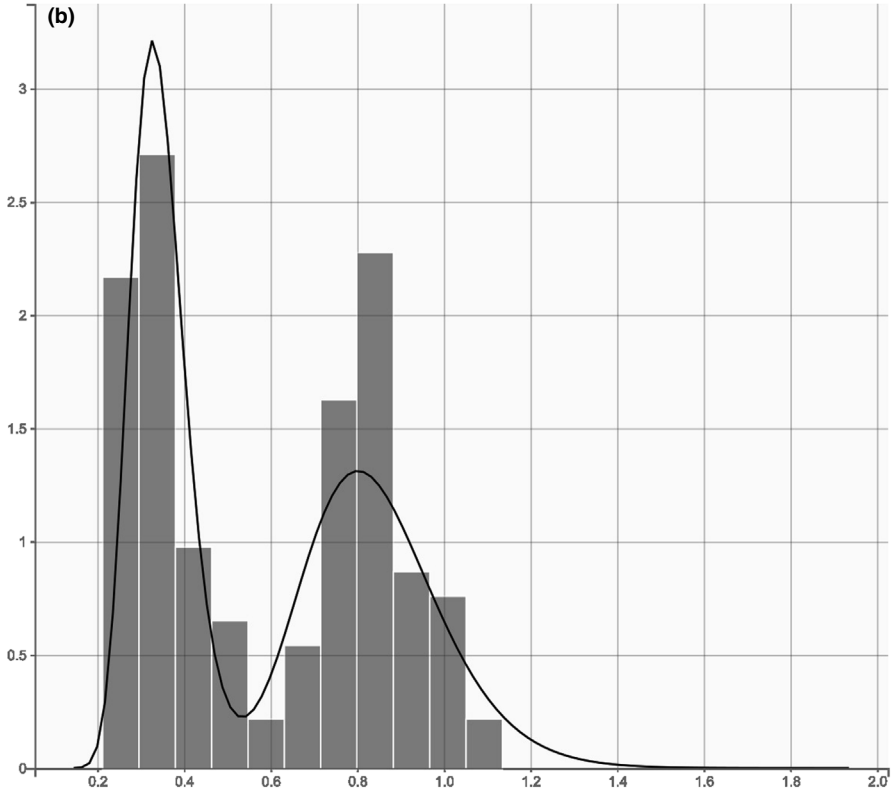


FIGURE 2 (a) Visual predictive check of the population pharmacokinetic model for doxycycline in plasma showing the empirical median, 10th and 90th percentiles (dotted lines) of the observed data (dots) falling within the prediction intervals generated by the model for these percentiles (white and gray areas). (b) True population distribution of the values of total body clearance (CL, black line) obtained with the final model with the categorical co-variate of age (lamb versus sheep) and the histogram of the empirical distribution of individual values of CL [Colour figure can be viewed at wileyonlinelibrary.com]

The development of the anatomy and function of the forestomach of maturing ruminants (de Backer & Bogaert, 1983) can result in considerable age-dependent pharmacokinetic variation. The values of Cmax and AUC_{0-t} were higher in lambs than in sheep, but the difference was not significant. Significantly lower value of AUMC can be a prerequisite for lower value of MRT in sheep than in lambs. However, prediction of $AUC_{0-\infty}$ with lower extrapolation than 20% is required to evaluate the age-dependent variations in MRT. Inter-individual variation in absorption resulted in variable T_{max} values, and a significant difference could not be demonstrated although

TABLE 3 Estimated population pharmacokinetic parameters for oral administration of doxycycline

Parameter	Fixed effect Mean ± SE	RSE (%)	Standard deviation of the random effects, ω Mean ± SE
Basic model without co-variates			
k_a (hr^{-1})	0.59	29.8	0.691 ± 0.219
V/*F* (L/kg)	6.40	24.9	0.740 ± 0.180
CL/*F* (L/kg hr^{-1})	0.47	21.3	0.494 ± 0.143
Residual error:			
a	0.083		
b	0.10		
Final model with a co-variate "adult"			
k_a (hr^{-1})	0.46	31.4	0.614 ± 0.194
V/*F* (L/kg)	5.57	25.4	0.692 ± 0.17
CL/*F* (L/kg hr^{-1})	0.34	14.4	0.221 ± 0.085
$ß_{CL/Fadult}$ (L/kg hr^{-1})	0.94	19.3	–
Residual error:			
a	0.11		
b	0.074		

Abbreviations: adult, co-variate for age with sheep = 1 and lamb = 0; CL, total body clearance; *F*, bioavailability; k_a, absorption rate constant; RSE, residual standard error; SE, standard error. $ß_{CL/Fadult}$, co-variate parameter adjusting the value of Cl for sheep; *V*, volume of distribution.

there was a tendency for later T_{max} values in lambs. A larger number of animals are required to prove significant differences in the absorption between lambs and sheep. The values of T_{max} in calves with immature rumen function (3.48 ± 0.63 hr) are similar to the data in lactating animals from our investigation and nonlactating sheep (3.43 ± 3.31 hr) (Castro et al., 2009; Meijer et al., 1993). These data show that information about doxycycline absorption in calves cannot be directly translated to lambs. Taken together, these results show that high inter-individual differences can be expected in the absorption of doxycycline in sheep. Possible explanation of the observed tendency for faster absorption of doxycycline in lactating sheep than in lambs is the type of feeding in these animals which depends on the development of rumen. The milk is still present in the diet of lambs, and probably, they still had milk in their stomachs at the start of treatment because the young animals were separated from their mothers before drug administration. Although the affinity of doxycycline to bind calcium ions is much lower than the other tetracyclines (de Fig ueiredo et al., 2019), such an interaction may cause delayed absorption.

Significantly higher doxycycline plasma concentrations in lambs than in sheep can be discussed in relation to maturation in the function of excretory organs such as liver and kidney with age. The values of AST and ALT in sheep depend on many factors and differ according to the breed, gender, pregnancy, lactation, and age (da Cruz et al., 2017; Ramos, Verde, Marca, & Fernández, 1994). In diseased animals, AST levels are indicative for kidney, muscle and heart diseases and to lesser extend to liver damages. Significant differences in the values of AST and ALT between lambs and sheep in our study are likely related to age and have been described in other studies (Abdel-Fattah, Hashem, Shaker, Ellamei, & Amer, 2013; da Cruz et al., 2017). The observed almost twofold higher levels of AST in lactating sheep can be related to higher muscle mass in mature animals and gradual increase in the metabolic activity of the enzyme with age (da Cruz et al., 2017). The ALT levels depend on liver function, and higher values in sheep than in lambs can be explained by age-dependent maturation of liver function and differences in the feed (Abdel-Fattah et al., 2013). The knowledge about the metabolism of doxycycline in animal species is scarce, and there are not strong evidences for the contribution of CYP450 enzymes in elimination of this antibiotic. Its metabolism in human being is not significant; however, enzyme inducers have been found to decrease the half-life of doxycycline (Holmes & Charles, 2009). Doxycycline has been identified as a substrate and modulator of the function of P-glycoprotein which can contribute to the differences in its elimination (Yücel, Değim, & Yilmaz, 2013). Age-related differences in doxycycline pharmacokinetics in sheep were further supported by the population pharmacokinetic analysis. A significant effect of the age on the apparent clearance (confounded by unknown bioavailability) was found, and dosing adjustment based on this co-variate is needed. Knowledge about higher values of apparent clearance of doxycycline in sheep can be used in a further study on doxycycline pharmacokinetics in lactating animals with a higher dose than the used in the current experiment.

Another source which contributes to the observed age-dependent differences in the pharmacokinetics of doxycycline is lactation of the sheep, included in this study. Although doxycycline administration is not permitted for lactating animals when the milk is intended for human consumption (Anonymous, 2015), it is indicated for a number of systemic infections in ruminants (Castro Robles et al., 2012; Ole-Mapenay & Mitema, 1997; Pudjiatmoko,

H., Ochiai, Y., Yamaguchi, T., & Hirai, K., 1998). Therefore, knowledge on doxycycline concentrations in milk is an important issue not only for regulatory purposes and also due to possibility suckling animals to receive significant amount of the antibiotic through the milk of treated sheep. Our results indicate that doxycycline penetrates into the milk of sheep which is in line with previously published data in cows, sheep, and goats (Jha et al., 1989; Ziv & Sulman, 1974). However, Ziv and Sulman (1974) did not provide data about the cows and sheep separately, and it is difficult to compare its disposition in milk of sheep. At last sampling point, 24 hr after administration, measurable concentrations of doxycycline were found in the milk and only traces of the antibiotic could be observed in plasma of the sheep in this study. The penetration of doxycycline in the milk was expected due to its lipophilicity (Riond & Riviere, 1988) and relatively high octanol/water partition coefficient (Franklin, Younis, & Myrdal, 2016). These properties of doxycycline result in the drug accumulating in fatty tissues, including the fat of the udder and ovine milk. A contribution of binding of doxycycline to calcium ions (de Fig ueiredo et al., 2019) in milk should be considered as a factor for high concentrations in milk. Excretion of the antibiotic through the milk of sheep should be taken into account when considering group treatment of sheep kept together with their offspring.

The recommended dose of 10 mg/kg suggests achieving effective plasma concentrations against *Anaplasma phagocytophilum*. Woldehiwet (2010) compared the activity of several antibiotics against five sheep strains of this pathogen. The value of MIC of doxycycline for all of them was 0.125 μg/ml (Woldehiwet, 2010). Its activity against *Anaplasma phagocytophilum* was greater than rifampin (MIC 0.5 μg/ml) and ciprofloxacin (MIC 1.0 μg/ml). The pathogen showed resistance to the rest of the tested drugs such as penicillin, ampicillin, ceftriaxone, streptomycin, and gentamicin (Woldehiwet, 2010). The percentage of binding to plasma proteins should always be taken into account when the data from in vitro tests have to be transferred to in vivo antimicrobial activity because only the free fraction of the drug is pharmacologically active. Doxycycline has shown high affinity for protein binding in ruminants when serum samples were analyzed: 90.2 ± 2.4% in sheep (Ziv & Sulman, 1974) and 92.3 ± 0.8% in cows (Riond et al., 1989). According to our data, after correction of the measured concentrations (described in Mileva, 2019), plasma levels in sheep will be higher than MIC of 0.125 μg/ml 14 hr after treatment or almost 60% from the dosing interval which suggest administration of higher doses. Better prediction of the efficacy of doxycycline in the cases of anaplasmosis requires measurement of the antibiotic concentrations in the white and red blood cells. One of the next steps in this research should therefore be to determine of the disposition of doxycycline in blood cells. Another step will be to use the population pharmacokinetic model developed in this study to predict the probability of attaining target values for pharmacokinetic/pharmacodynamic indices for different indications in order to identify the most appropriate dosage regimens in sheep and lambs.

ACKNOWLEDGMENTS

The authors would like to thank to Certara USA, Inc. for software license provided through their Center of Excellence program.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

RM and AM read and approved the final manuscript and contributed to study design, sample collection, data analysis, and manuscript preparation and review. SS read and approved the final manuscript and contributed to sample collection. RG read and approved the final manuscript and designed and performed the population pharmacokinetic analysis, and manuscript preparation and review.

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How to cite this article: Mileva R, Subev S, Gehring R, Milanova A. Oral doxycycline pharmacokinetics: Lambs in comparison with sheep. *J vet Pharmacol Therap.* 2020;43:268–275. https://doi.org/10.1111/jvp.12859