Residue study of doxycycline and 4-epidoxycycline in pigs medicated *via* drinking water†

Siska Croubels,*[a] Kris Baert,[a] Jul De Busser[b] and Patrick De Backer[a]

[a] *Department of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, University of Gent, Salisburylaan 133, B-9820 Merelbeke, Belgium*
[b] *Kela Laboratoria n.v., St. Lenaartseweg 48, B-2320 Hoogstraten, Belgium*

Received 29th June 1998, Accepted 15th October 1998

A study was performed to determine the residues in edible tissues of healthy pigs after continuous administration of doxycycline with drinking water for five consecutive days at a dose rate of 10.5 mg doxycycline kg^{-1} body weight (BW) per day. Quantitation was performed using a validated HPLC method with fluorescence detection. The method was able to separate doxycycline and its 4-epimer, 4-epidoxycycline. This epimer was found in kidney, liver, skin, fat and muscle tissue. The method was validated at the maximum residue limit (MRL), at half the MRL and at double the MRL for both doxycycline and 4-epidoxycycline. Linear calibration curves were obtained in spiked tissues ($r > 0.99$). The accuracy of the calibrators of the calibration curves was within −20% to +10%. The accuracy and precision (expressed as the within-run repeatability) were found to be within the required ranges for the specific concentration. The limits of detection and limits of quantification were below one-half of the MRL. The quantification limits were 50 μg kg^{-1} for doxycycline and 100 μg kg^{-1} for 4-epidoxycycline in kidney and liver, 20 μg kg^{-1} for doxycycline and 50 μg kg^{-1} for 4-epidoxycycline in skin and fat and 10 μg kg^{-1} for doxycycline and 50 μg kg^{-1} for 4-epidoxycycline in muscle tissue. The withdrawal time was calculated according to the recommendations of the European Agency for the Evaluation of Medicinal Products (EMEA/CVMP/036/95) and was set at 3 days. The plasma concentration of doxycycline and the stability of doxycycline in drinking water were also determined during the treatment period. The mean plasma concentration of doxycycline during the treatment period ranged from 0.83 to 0.96 μg ml^{-1}. Thirty-six hours after the withdrawal from medicated drinking water, no plasma levels could be detected. Samples of medicated water were taken at time zero and at 24 h after addition of doxycycline to the drinking water. No statistically significant difference in the mean drinking water concentration was seen at time zero and at time 24 h (Student's *t*-test, $\alpha = 0.05$).

Introduction

Doxycycline (DOX) is a broad-spectrum antibiotic which is widely used in the treatment of respiratory tract infections in various species. The European Union (EU) legislation on veterinary drug residues established, in 1997, provisional maximum residue limits (MRL) for DOX in pigs, including its 4-epimer (4-EDOX), at 600 μg kg^{-1} in kidney, 300 μg kg^{-1} in liver, skin and fat and 100 μg kg^{-1} in muscle.[1] 4-EDOX is an antibacterially inactive epimer of DOX. Quantification therefore required the use of a method that was capable of separating DOX and 4-EDOX in tissues.[2] However, after this study had been conducted, a definite MRL for DOX became available in 1998 in which the 4-epimer was no longer included in the marker residue.[3] Residue studies of DOX have been conducted in several species, including chickens,[4] turkeys,[2] calves[5] and pigs.[6] The purpose of this study was to determine the residues of DOX and 4-EDOX in edible tissues (kidney, liver, skin, fat and muscle) of pigs after 5 days of oral medication *via* drinking water of 10.5 mg DOX kg^{-1} body weight (BW) per day. The DOX concentrations in plasma and the stability of DOX in drinking water were also determined. Based on the tissue residues, a withdrawal time was calculated according to Guideline No. EMEA/CVMP/036/95 of the Committee for Veterinary Medicinal Products.[7]

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

Experimental

Animals

The study was conducted with 20 (10 females and 10 castrated males) healthy Belgian Landrace stress-negative pigs weighing 18.2–27.9 kg at the start of the experiments. The pigs were housed in two groups of four animals (two females and two castrated males) and two groups of five animals; one group of two pigs (one female and one castrated male) was kept as blanks. The animals were fed a pig feed free from antibiotics. Food and water were available *ad libitum*. The drinking water system consisted of drinking nipples per box connected *via* plastic piping to a plastic storage tank of 100 l. The tanks were provided with a continuous stirring system and provisions for the measurement of daily water intake.

Experimental design

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of the University of Gent (No. 97/14). After a controlled drug free period of 8 days, medicated drinking water was given for a period of five consecutive days at a daily dose of 10.5 mg DOX kg^{-1} BW (doxycycline 75%, Kela, Hoogstraten, Belgium). Pigs were weighed daily and the water intake per group was recorded daily. Based on the mean daily water intake and the mean body weight per group of four

or five pigs, the dosage of DOX was calculated every day for medication of 50 l.

Samples of medicated water in the tank of one box were taken at time zero (after homogenisation of the solution) and after 24 h to control the stability of DOX. The samples were analysed within 1 h after sampling.

During the treatment period, blood samples from groups 1 and 2 (n = 8) were collected from the jugular vein by venipuncture. Samples (±10 ml) were taken before (0 h) and at 12, 36, 60, 84, 108, 132 and 156 h after initiation of the trial. Blood samples were centrifuged immediately and the plasma was stored at −20 °C pending analysis.

The pigs were sacrificed at 3 (n = 4), 7 (n = 4), 11 (n = 5) and 15 (n = 5) days after cessation of medication. One whole kidney and about 100 g of liver, skin, fat and muscle were collected separately to avoid contamination and frozen at −20 °C pending analysis. All samples were analysed within 2 months after sampling.

Analytical methods and validation

General. The DOX concentrations in plasma were measured according to the method described by Nielsen and Gyrd-Hansen[8] for oxytetracycline, with the exception that the method used for this study included an internal standard. Tissue concentrations of DOX and 4-EDOX were determined as described previously using the same sample preparation procedure.[9] Both methods use an internal standard (IS), demethylchlortetracycline (DMCTC), which is added to the homogenised tissues, plasma or drinking water just before sample preparation.

Reference substances. DOX was a Chemical Reference Substance (CRS) of the European Pharmacopoeia (Council of Europe, Strasbourg, France). 4-EDOX.hyclate was a gift from the Laboratory of Pharmaceutical Technology (Faculty of Pharmaceutical Sciences, University of Gent, Belgium) and DMCTC.HCl (IS) was from Fluka Chemie (Buchs, Switzerland).

Chromatography. The HPLC system consisted of a gradient HPLC pump (Model P4000, Thermo Separation Products (TSP), San Jose, CA, USA), an autosampler kept at 15 °C (Model AS3000, TSP) and a reversed-phase polymeric column type PLRP-S (8 μm, 250 × 4.6 mm id, kept at room temperature) in combination with a PLRP-S guard cartridge of 5 × 3.0 mm (Polymer Laboratories, Church Stretton, UK).

For determinations in plasma, detection was based on UV measurements at λ = 356 nm (UV detector Model UV1000, TSP). The mobile phase was prepared with HPLC-grade solvents and contained 0.01 M oxalic acid in water (A) and acetonitrile (B). A gradient solvent programme was run: 0–4 min (A : B, 85 : 15); 4–20 min (A : B, 60 : 40, linear gradient); 20–21 min (A : B, 85 : 15); at 30 min the next injection was performed. The flow rate was 1 ml min^{-1} and the injection volume was 100 μl.

For tissue samples, fluorescence was measured after post-column addition of 5% (m/v) zirconyl chloride octahydrate in HPLC-grade water. The excitation wavelength was set at 406 nm, while emission was measured at 515 nm. The post-column configuration consisted of a Model P1000 inert isocratic pump (TSP), a mixing tee, a Waters reactor coil (Waters Chromatography, Milford, MA, USA) and a Model FP-920 fluorescence detector (Jasco, Tokyo, Japan). Detector signals were processed using PC1000 software, version 2.5 (TSP). The mobile phase contained 0.01 M oxalic acid in water (A), acetonitrile (B) and methanol (C). A gradient solvent programme with the following conditions was run: 0–5 min (A : B : C, 80 : 15 : 5); 5–20 min (A : B : C, 40 : 20 : 40, linear gradient); 20–25 min (A : B : C, 80 : 15 : 5); at 35 min the next injection was performed. The flow rate was 1 ml min^{-1} and was identical for both HPLC pumps. The injection volume was 100 μl for the analysis of muscle tissue, 20 μl for kidney and liver tissue and 50 μl for skin and fat tissue.

Naturally contaminated muscle tissue and spiked plasma were used as quality control (QC) samples. The QC samples served as a control to check the acceptance of the extraction procedure and the HPLC run.

Validation. The methods were validated for DOX and 4-EDOX by a set of parameters which are in compliance with EU requirements.[10] The linearity of the assays was checked using spiked blank tissues, tap water or plasma with spike levels including the MRL. For each calibration curve, four or six levels were used, including a zero level. Calibration curves were obtained using the least squares regression procedure of the peak area ratio (DOX/IS) *versus* concentration ratio (DOX/IS). The accuracy was defined as the closeness of agreement between the true (spike) value and the mean result of a series of experiments (n = 6). It was determined by comparing the measured concentration to the spiked concentration. In this validation procedure, the term precision covers the repeatability and is expressed as the relative standard deviation (RSD, %). The accuracy and precision were evaluated at the MRL, at half the MRL and at double the MRL for all tissues and for both DOX and 4-EDOX. The limit of detection (LOD) was defined as three times the peak-to-peak noise determined at the retention time of DOX and 4-EDOX. The limit of quantification (LOQ) was defined as the lowest concentration for which the method is validated with an accuracy and precision that fall within the ranges recommended by the EU.

Results and discussion

Chromatography

Fig. 1 shows the chromatograms of a blank pig liver sample (A) and of an extract of a pig liver sample 3 days after administration of 5 days of 10.5 mg DOX kg^{-1} BW per day with drinking water (B). The analysis of blank tissue samples showed the absence of endogenous peaks co-eluting with the IS, DOX and 4-EDOX. Although the resolution of 4-EDOX from DOX was poor, all chromatograms of the tissues of the pigs slaughtered at 3 days showed detectable peaks of 4-EDOX.

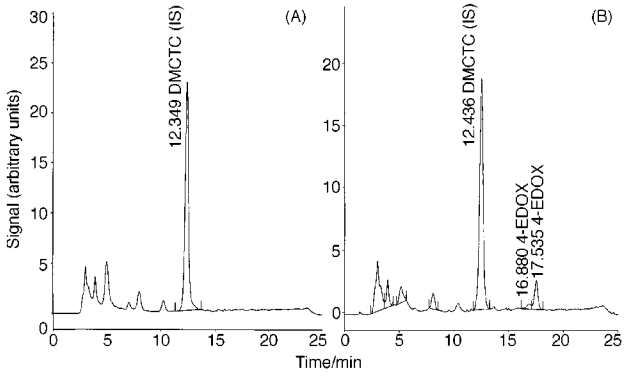

Fig. 1 (A) Chromatogram of a blank pig liver sample. (B) Chromatogram of an extract of a pig liver sample 3 days after cessation of medication (10.5 mg doxycycline kg^{-1} BW in drinking water for five consecutive days). Conditions: column, PLRP-S 8 μm (250 × 4.6 mm id) with a 5 × 3.0 mm PLRP-S guard cartridge; mobile phase, 0.01 M oxalic acid–acetonitrile–methanol gradient; flow rate, 1 ml min^{-1}; fluorescence detection, λ_{ex} = 406 nm, λ_{em} = 515 nm.

Validation

Linear calibration curves of DOX were obtained in tap water (0–50 μg ml^{-1}; r = 0.9999), plasma (0–1 μg ml^{-1}; r > 0.9976), kidney tissue (0–1500 μg kg^{-1}; r = 0.9969), liver, skin and fat tissue (0–500 μg kg^{-1}; r > 0.9996) and muscle (0–300 μg kg^{-1}; r = 0.9998). Linearity was also shown for 4-EDOX in the different tissues for the same levels as DOX (r > 0.9973). The accuracy of the calibrators of the calibration curves was within −20% to +10%.

The accuracy for the determinations in plasma was evaluated at 0.2 and 40 μg ml^{-1}. For tissues, the concentrations examined were equal to the MRL, half the MRL and double the MRL. The accuracy for these measured concentrations was within acceptable limits for both DOX and 4-EDOX, which are set at −20% to +10% for concentrations exceeding 10 μg kg^{-1}. The precision was evaluated at the same levels as the accuracy parameter. The maximum allowable tolerances for the imprecision (RSD_{max}, %) for analyses carried out under repeatability conditions are one-half to two-thirds of the values calculated according to the Horwitz equation.[11] The obtained RSD values (%) for both DOX and 4-EDOX imprecision in plasma and tissues were below the calculated RSD_{max} values.

The LOD and LOQ were below one-half of the MRL. The LOD were 0.05 μg ml^{-1} DOX for plasma, 10.9 μg kg^{-1} DOX and 46.2 μg kg^{-1} 4-EDOX for kidney, 8.8 μg kg^{-1} DOX and 42.9 μg kg^{-1} 4-EDOX for liver, 2.4 μg kg^{-1} DOX and 10.7 μg kg^{-1} 4-EDOX for skin, 2.4 μg kg^{-1} DOX and 15.0 μg kg^{-1} 4-EDOX for fat and 1.3 μg kg^{-1} DOX and 10.7 μg kg^{-1} 4-EDOX for muscle tissue. The LOQ were 0.2 μg ml^{-1} DOX for plasma, 50 μg kg^{-1} DOX and 100 μg kg^{-1} 4-EDOX for kidney and liver, 20 μg kg^{-1} DOX and 50 μg kg^{-1} 4-EDOX for skin and fat and 10 μg kg^{-1} DOX and 50 μg kg^{-1} 4-EDOX for muscle tissue. At each level, the accuracy and precision fell within the required ranges for that specific concentration.

The specificity of the method was shown since no interfering peaks were obtained with the same retention time as DOX, 4-EDOX and the IS in the chromatograms of blank samples. Moreover, no interference from other tetracycline antibiotics, such as oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC), was seen in the chromatogram. The capacity factors k were 2.0, 2.9, 4.1, 5.1, 5.8 and 6.0 for OTC, TC, DMCTC (IS), CTC, 4-EDOX and DOX, respectively.

Doxycycline drinking water concentration

The mean drinking water concentrations were 86 ± 16.7 μg ml^{-1} (n = 5) and 88 ± 14.9 μg ml^{-1} (n = 5) at 0 h and 24 h after addition to the tank, respectively. A statistical Student's t-test (α = 0.05) was performed to monitor the 24 h stability. Therefore, an F-test was carried out first to test whether the variances σ^2 at 0 h and 24 h differed significantly (α = 0.05). The t-test showed no statistically significant difference in concentration at 0 h and 24 h. It can be concluded that DOX was stable during 24 h in tap water.

Doxycycline plasma concentration

The mean plasma concentration of eight pigs during the treatment period ranged from 0.83 to 0.96 μg ml^{-1}. These levels are above the minimal inhibitory concentrations (MIC_{50}) of different pathogenic organisms. These MIC_{50} values range from 0.06 to 0.25 μg ml^{-1} for *Streptococcus suis*, from 0.25 to 0.5 μg ml^{-1} for *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* and from 0.25 to 1.0 μg ml^{-1} for *Bordetella bronchiseptica*.[12] Bousquet *et al.*[13] reported a MIC_{50} value of 0.5 μg ml^{-1} for *Actinobacillus pleuropneumoniae*. Thirty-six hours after the withdrawal from medicated drinking water, no plasma levels could be detected.

Doxycycline and 4-epidoxycycline tissue concentrations

In Table 1, the mean tissue concentrations and their SD values of kidney, liver, skin, fat and muscle at 3 (n = 4), 7 (n = 4), 11 (n = 5) and 15 (n = 5) days after cessation of medication are presented. The concentrations in all matrices were below the MRL at 3 days after cessation of medication and below the respective LOQ at 11 days after cessation of medication. The linearity of the plot of log concentration *versus* time indicated that the residue depletion fitted a one compartment model. Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time is determined as the time when the one-sided, 95% upper tolerance limit of the regression line with a 95% confidence level is below the MRL. The European Agency for the Evaluation of Medicinal Products Guideline recommends that values less than the LOQ should be set at one-half of the LOQ. Using this approach, the withdrawal time could only be calculated for liver tissue: 3 days. The concentrations in the other tissues were too far below the MRL to calculate a withdrawal time, even at 3 days after cessation of medication. Anadón *et al.*[6] found that a withdrawal time of 6 days was necessary after intramuscular administration to pigs of 10 mg DOX kg^{-1} BW for 4 days. A possible explanation could be local irritation at the injection site associated with persistent DOX residues. In turkeys, we found a withdrawal time of 17 days after administration of 25 mg DOX.HCl kg^{-1} BW *via* drinking water for four consecutive days,[2] indicating species dependent elimination.

A previous residue study of DOX in turkeys revealed the presence of the 4-epimer in liver and muscle tissue.[2] In this bird

Table 1 Mean tissue concentrations of doxycycline and 4-epidoxycycline in kidney, liver, skin, fat and muscle at 3 (n = 4), 7 (n = 4), 11 (n = 5) and 15 (n = 5) days after cessation of medication (10.5 mg doxycycline kg^{-1} BW in drinking water for five consecutive days)

		Kidney/μg kg^{-1}		Liver/μg kg^{-1}		Skin/μg kg^{-1}		Fat/μg kg^{-1}		Muscle/μg kg^{-1}	
	Day	DOX	4-EDOX	DOX	4-EDOX	DOX	4-EDOX	DOX	4-EDOX	DOX	4-EDOX
Mean	3	104	107	55	186	25	<LOQ	<LOQ	<LOQ	24	<LOQ
s		33.0	6.6	8.5	30.3	3.7	—	—	—	4.9	—
RSD		31.6	6.2	15.4	16.3	15.1	—	—	—	21.0	—
Mean	7	62	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	15	<LOQ
s		10.5	—	—	—	—	—	—	—	4.0	—
RSD		17.0	—	—	—	—	—	—	—	26.4	—
Mean	11	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
s		—	—	—	—	—	—	—	—	—	—
RSD		—	—	—	—	—	—	—	—	—	—
Mean	15	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
s		—	—		—	—	—	—	—	—	—
RSD		—	—	—	—	—	—	—	—	—	—

species, it was likely that the epimer was formed *in vivo*. This was based on the fact that all chromatograms of the spiked tissues showed no detectable peak eluting at the retention time of 4-EDOX, whereas all chromatograms of the incurred turkey tissues showed the presence of a peak at the retention time of 4-EDOX. Moreover, both the spiked and the incurred samples were analysed the same day using the same method. This indicates that the 4-epimer was probably formed *in vivo* and not during sample preparation. This study in pigs medicated *via* drinking water also showed the presence of the 4-epimer in different incurred tissues, indicating again the possible formation of the metabolite *in vivo* in the pig.

Acknowledgements

The authors thank Kela n.v. (Hoogstraten, Belgium) for funding the study. Miss M. Geerinck is acknowledged for preparing the samples prior to HPLC analysis and Mrs E. Raulo-Roelens for typing the manuscript.

References

1 Commission Regulation (EC), 17/97, 1997, No. L5/12.
2 S. Croubels, H. Vermeersch, P. De Backer, M. D. F. Santos, J. P. Remon and C. Van Peteghem, *J. Chromatogr. B*, 1998, **708**, 145.
3 Commission Regulation (EC), 1570/98, 1998, No. L205/10.
4 A. Anadón, M. R. Martínez-Larrañaga, M. J. Díaz, P. Bringas, M. C. Fernández, M. L. Fernandez-Cruz, J. Iturbe and M. A. Martinez, *Avian Pathol.*, 1994, **23**, 79.
5 E. W. van Dongen and J. F. M. Nouws, *Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, May 3–5, 1993*, ed. N. Haagsma, A. Ruiter and P. B. Czedik-Eysenberg, University of Utrecht, Faculty of Veterinary Medicine, 1993.
6 A. Anadón, M. R. Martínez-Larrañaga, M. L. Fernandez-Cruz, M. J. Díaz, R. Fernandez, B. Sevil and R. Anton, *Proceedings of the EuroResidue III Conference on Residues of Veterinary Drugs in Food, Veldhoven, The Netherlands, May 6–8, 1996*, ed. N. Haagsma and A. Ruiter, University of Utrecht, Faculty of Veterinary Medicine, 1996.
7 The European Agency for the Evaluation of Medicinal Products, *Note for Guidance: Approach Towards Harmonisation of Withdrawal Periods*, No. EMEA/CVMP/036/95, 1995.
8 P. Nielsen and N. Gyrd-Hansen, *J. Vet. Pharmacol. Therap.*, 1996, **19**, 305.
9 S. Croubels, K. Vanoosthuyze and C. Van Peteghem, *J. Chromatogr. B*, 1997, **690**, 173.
10 Commission Decision No. 93/256, 1993, No. L118.
11 W. Horwitz, L. R. Kamps and K. W. Boyer, *J. Assoc. Off. Anal. Chem.*, 1980, **63**, 1344.
12 A. Pijpers, B. Van Klingeren, E. J. Schoevers, J. H. M. Verheijden and A. S. J. P. A. M. Van Miert, *J. Vet. Pharmacol. Therap.*, 1989, **12**, 267.
13 F. Bousquet, H. Morvan, I. Aitken and J. H. Morgan, *Vet. Rec.*, 1997, **141**, 37.

Paper 8/04936J