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Liquid chromatographic separation of doxycycline and 4-epidoxycycline in a tissue depletion study of doxycycline in turkeys

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Abstract

Liver and muscle tissue residues of doxycycline in turkeys were determined following administration of 25 mg doxycycline·HCl/kg BW in the drinking water under field conditions. 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 both liver and muscle tissue. The detection limits of the method were estimated at 1.2 ng/g and 1.0 ng/g of doxycycline in liver and muscle tissue, respectively, using a signal-to-noise ratio of 3:1. The recovery of doxycycline was determined from spiked tissues and was 63±3.8% and 66±3.1% for liver and muscle, respectively (n=6). Within-day and between-day imprecision, expressed as the R.S.D. was below 7.4%. Linear calibration curves (r>0.997) were obtained in spiked liver between 0 and 1500 ng/g and in spiked muscle between 0 and 500 ng/g. A good stability of doxycycline was observed in liver samples after storage for 22 days at −20°C. The correlation between the residues in the liver and the muscle was expressed as the correlation coefficient r and was 0.9884. The depletion kinetics of doxycycline fitted a one-compartment model. The elimination half-life ($T_{1/2}$) of doxycycline was 77.7 h and 78.0 h in muscle and liver, respectively. Furthermore, the residue depletion kinetics were used to establish a withdrawal period in conformity with official guidelines. The withdrawal times necessary to reach concentrations below maximum residue limits (MRLs), as imposed by the EU, were 12 days and 17 days for liver and muscle tissue, respectively.

Keywords: Doxycycline; 4-Epidoxycycline

1. Introduction

Tetracycline antibiotics are often used in the treatment of avian infectious diseases, especially in bacterial respiratory tract diseases. Within this group of antibiotics, doxycycline (DOX) is widely used due to its advantageous pharmacokinetic properties. These include its better bioavailability from the

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gastrointestinal tract, its stronger lipophilic character and its higher affinity for tissues. However, the higher lipid solubility of DOX may result in higher and more persistent residue levels in edible tissues. The EU legislation on veterinary drug residues has laid down a provisional maximum residue limit (MRL) for DOX in poultry, including its 4-epimer (4-EDOX), at 600 ng/g in kidney, at 300 ng/g in liver and skin with adhering fat and at 100 ng/g in muscle [1]. 4-EDOX is an antibacterially inactive epimer of DOX which is formed spontaneously in an acidic medium (pH 2–6) [2]. This requires the development of methods that are capable in separating DOX and 4-EDOX in tissues. Only few such methods are available [3], mostly describing the purity control of doxycycline standard samples [4].

Pharmacokinetic and bioavailability studies of DOX have been conducted in several avian species including pigeons [5], chickens [6] and turkeys [7,8]. All these studies were conducted under laboratory conditions. However, only one recent study reports on experiments carried out to determine DOX plasma levels in turkeys medicated under field conditions [9]. After this field experiment, the residues of DOX in edible tissues were determined. Skin with adhering fat was only included as marker tissue since January 1997 [1]. This study was conducted in 1996, before the latter MRL was published, which means that skin was not analysed. Also, kidney tissue was not analysed since only these tissues most likely consumed were chosen.

The purpose of this study was to determine the residues of DOX in edible tissues of turkeys, i.c. liver and muscle tissue, after medication via drinking water at a concentration of 25 mg DOX·HCl/kg BW under field conditions. Based on the results, a withdrawal time was calculated according to the recent guideline No. EMEA/CVMP/036/95 of the Committee for Veterinary Medicinal Products [10]. The stability of DOX in liver tissue was also determined.

2. Experimental

2.1. Animal experiments

The field experiment was conducted in a turkey farm (Nazareth, Belgium) housing 2100 six-week-old healthy male turkeys weighing 1.4±0.2 kg (mean±S.D.) at the start of the experiment. Medicated drinking water was given for a period of four consecutive days at a total daily dose of 25 mg DOX·HCl/kg BW. Food and water were available ad libitum. The drinking water system consisted of individual drinking bowls connected via plastic piping to a 120-l plastic storage tank. The level in the tank was kept constant by means of a floating-gauge. A light cycle of 24 h was maintained in the stable and the temperature was kept constant within a range of 20–23°C. At 7 a.m. and 7 p.m. a mixture of 40 g DOX·hyclate (DOX·hemiethanolate hemihydrate, α-Pharma, Zwevegem, Belgium) and 120 g anhydrous citric acid (Cerestar Bioproducts, Sas Van Gent, The Netherlands) was added to the tank as a concentrated solution and homogenized. The addition of 0.1% (m/v) anhydrous citric acid was necessary for the solubility and stability of DOX in tap water [9].

Six turkeys were taken at random and sacrificed before the start of the experiment (control group) and 12, 36, 84, 180, 252 and 324 h after the last DOX administration. Breast muscle and liver samples were collected separately to avoid mutual contamination and frozen at −20°C pending analysis. The analysis was carried out within two months after sampling.

2.2. Analytical method

The DOX concentration in both liver and muscle tissue was determined using a high-performance liquid chromatography (HPLC) method with fluorescence detection and demethylchlortetracycline (DMCTC) as internal standard (I.S.).

2.2.1. Chromatography

The chromatographic system consisted of a gradient HPLC pump (Waters Model 600-MS, Waters Chromatography, Milford, MA, USA), a reversed-phase (RP) polymeric column type PLRP-S (8 μm, 250×4.6 mm I.D., kept at room temperature) in combination with a PLRP-S guard cartridge of 5× 3.0 mm (8 μm, Polymer Laboratories, Church Stretton, UK) and a Waters Model 470 fluorescence spectrophotometer. Fluorescence was measured after post-column addition of 5% (m/v) zirconyl chloride octahydrate in HPLC-grade water [11]. The post-column reagent was kept in an amber coloured bottle

and an ice-bath for stability reasons. The excitation wavelength was set at 406 nm while emission was measured at 515 nm. The post-column configuration consisted of a Waters Model 610 isocratic pump, a mixing tee U-466 (Upchurch Scientific, Oak Harbor, WA, USA) and a Waters reactor coil of 600 μl. Detector signals were processed with a HP 3396 Series II Integrator (Hewlett-Packard, Waldbronn, Germany). The mobile phase was prepared with HPLC-grade solvents (BDH, Poole, UK) and contained 0.01 *M* oxalic acid in water (A), acetonitrile (B) and methanol (C). A gradient solvent programme with following conditions was run: 0–5 min (A–B–C, 80:15:5, v/v/v); 5–20 min (A–B–C, 40:20:40, linear gradient); 20–25 min (A–B–C, 80:15:5) and at 35 min next injection. These conditions made it possible to separate DOX and 4-EDOX. The flow-rate was 1.0 ml/min and was identical for both HPLC pumps. All DOX-containing solutions were protected from light.

2.2.2. Chemicals

The same batch of DOX·hyclate (α-Pharma) as was administered to the turkeys was used for the calibration curves. 4-EDOX·hyclate was a gift from the Laboratory of Pharmaceutical Technology, University of Ghent, Belgium. 6-Epidoxycycline (6-EDOX) and metacycline (MTC) were CRSs (chemical reference substances) from the European Pharmacopoeia, Strasbourg, France.

2.2.3. Sample preparation

The sample preparation was as described previously [12]. Three grams of minced tissues were placed in a centrifuge tube. The validation samples were spiked at this point with working standard solutions of the I.S. and DOX. The contents were homogenized with 20 ml of 0.1 *M* sodium succinate buffer (pH 4.0) and shaken for 10 min on a horizontal shaker. Next, 20 ml of methanol were added, followed by ultrasonication for 5 min and centrifugation for 10 min at 2666 *g* at 4°C. The clean-up of the filtered supernatant was done with the metal chelate affinity chromatography (MCAC) technique, as described elsewhere [13]. Further concentration of the MCAC eluate was performed on an Empore extraction membrane with RP and cation-exchange properties, type SDB-RPS [poly(styrene–divinylbenzene)-RP sulfonated], 3M Company (St. Paul, MN, USA) marketed by Varian (Harbor City, CA, USA). After application of the pH-adjusted MCAC eluate, the membrane was washed with 1 ml of 0.1 *M* HCl and DOX was eluted with 4×250 μl of HPLC-grade methanol containing concentrated (25%) ammonia (97:3, v/v). The extract was evaporated to dryness under nitrogen (40°C). The dried sample was reconstituted with 250 μl of 0.01 *M* oxalic acid in HPLC-grade water, vortexed and ultrasonicated. For the study of the recovery efficiency, DMCTC was added at this stage as an external standard: the peak-area ratio of DOX/DMCTC was compared with the corresponding ratio from a calibration standard solution. A 100-μl aliquot was injected onto the HPLC system.

3. Results and discussion

3.1. Chromatography

Initially, a mobile phase containing 0.01 *M* oxalic acid in water and acetonitrile, as described elsewhere [12] was used for the chromatography of DOX. However, the presence of an interfering peak in the chromatograms of the incurred turkey tissues required an adaptation of the HPLC gradient conditions. After addition of methanol to the mobile phase, as described in Section 2.2.1, a better separation was achieved. The interfering compound seen in the incurred tissues could be a degradation or by-product formed during the sample preparation or formed in vivo. Possible degradation products were epimers of DOX, i.c. 6-EDOX and 4-EDOX. A possible by-product was metacycline (MTC) since DOX is produced synthetically out of MTC, resulting in the possible presence of traces of MTC in the DOX raw material [14]. The identification of the compound seen in the incurred samples was based both on its retention characteristics and on standard addition to the MCAC eluates of incurred liver samples. Table 1 shows the capacity factors *k* for DOX and its major degradation or by-products without addition of methanol and after addition of methanol to the mobile phase. The capacity factor *k* was identical for 4-EDOX standard and for the compound seen in the incurred samples. Moreover, a small amount of 4-EDOX and 6-EDOX was added to the MCAC eluates of incurred liver samples. Fig. 1

Table 1
Capacity factors k for doxycycline and its major degradation or by-products without addition of methanol and after addition of methanol to the mobile phase

	With addition of CH_3OH	Without addition of CH_3OH
6-EDOX	5.70	4.71
MTC	5.80	4.80
4-EDOX	5.96	4.80
DOX	6.16	4.97

shows the chromatograms of an extract of a turkey liver sample after administration of 25 mg DOX·HCl/kg BW with the drinking water (a), of the same extract after addition of 4-EDOX (b) and of another extract of an incurred liver sample after addition of 6-EDOX (c). Fig. 1b shows the increase in peak area of 4-EDOX. Fig. 1c shows the separation between 6-EDOX and 4-EDOX. The chromatogram of the same extract as in Fig. 1c but before addition of 6-EDOX showed no peak eluting with the same retention time as 6-EDOX, illustrating the selectivity of the method.

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. Since the same batch of DOX·hyclate as was administered to the turkeys was used for spiking, the presence of the 4-epimer in the incurred turkey tissues was not caused by an impurity in the batch of DOX·hyclate. This indicates that the 4-epimer was probably partially formed in vivo and to a lesser extent during the sample preparation. However, additional studies are necessary to confirm this hypothesis. Moreover, the 4-EDOX peak was detected in both liver and muscle tissue, but to a lesser extent in the latter. The 4-EDOX was not quantified, since at the time of this study 4-EDOX was not yet included as marker residue in the MRL [1]. Information on the in vivo metabolism of DOX is scarce. Conflicting information obtained by chromatographic and mass spectrometric analysis exists in the literature concerning DOX biotransformation. Other older tetracyclines, such as oxytetracycline, tetracycline and chlortetracycline are known to be metabolically inert [15]. The lack of metabolites of DOX was claimed in humans [3] and pigs [16], whereas a metabolite was identified in liver and kidney tissue of rats and mice and in urine of humans [17]. Riond and Riviere [18] related this controversy to the presence of 4-EDOX which appears spontaneously in acidic medium or to other artefacts. Nelis and De Leenheer [3] also found minor amounts of 4-EDOX in urine of humans who ingested DOX. Its presence was attributed to the weakly acidic medium of the urine. 4-EDOX was barely detectable in faeces, explained in terms of the less favorable pH medium in the intestine. Possible epimerisation of DOX in the drinking water due to the acidic pH of the drinking water during the animal experiment was excluded. Experiments carried out at the Laboratory of Pharmaceutical Technology showed no increase in the epimer after 48 h kept at 30°C [19].

3.2. Validation of the analytical method

3.2.1. Selectivity

Fig. 2 shows a chromatogram of a pool of blank turkey liver samples ($n=6$) (a) and of blank turkey liver spiked with DOX (600 ng/g) (b). No interference of endogenous compounds was detected for DOX, as shown in Fig. 2a. Interference of DOX with the I.S. or degradation and by-products (4-EDOX, 6-EDOX and MTC) was not seen, as described in Section 3.1. The purity of the DOX peak was verified using a diode array detector (Waters Model 991). A liver sample obtained from a turkey given medicated drinking water and DOX standard were analysed. The DOX peak was checked for identity by comparison of the UV spectra (230–370 nm). The spectra revealed the same characteristics, so the proposed method is selective for DOX.

3.2.2. Analytical recovery and precision

The extraction recoveries obtained at the actual MRL were 63±3.8% (R.S.D.=6.0%; $n=6$) and 66±3.1% (R.S.D.=4.7%; $n=6$) for liver and muscle,

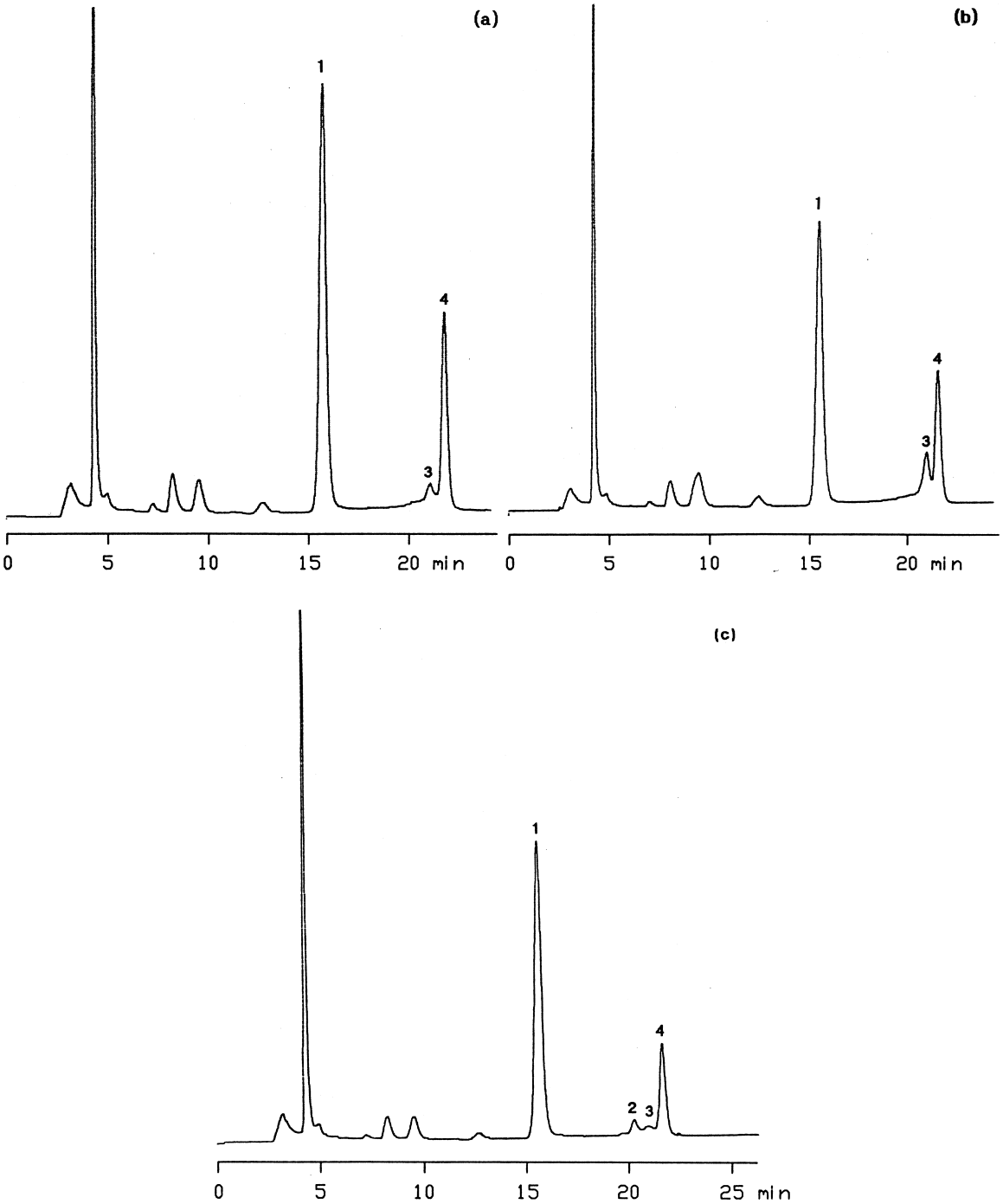

Fig. 1. (a) Chromatogram of an extract of a turkey liver sample after administration of 25 mg DOX·HCl/kg BW with the drinking water. (b) Chromatogram of the same extract after addition of 4-EDOX. (c) Chromatogram of another extract of an incurred liver sample after addition of 6-EDOX. Peaks: 1=DMCTC (I.S.), 2=6-EDOX, 3=4-EDOX and 4=DOX. Conditions: column, PLRP-S 8 μm (250×4.6 mm I.D.) 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; fluorescence detection, λ_{ex}=406 nm, λ_{em}=515 nm.

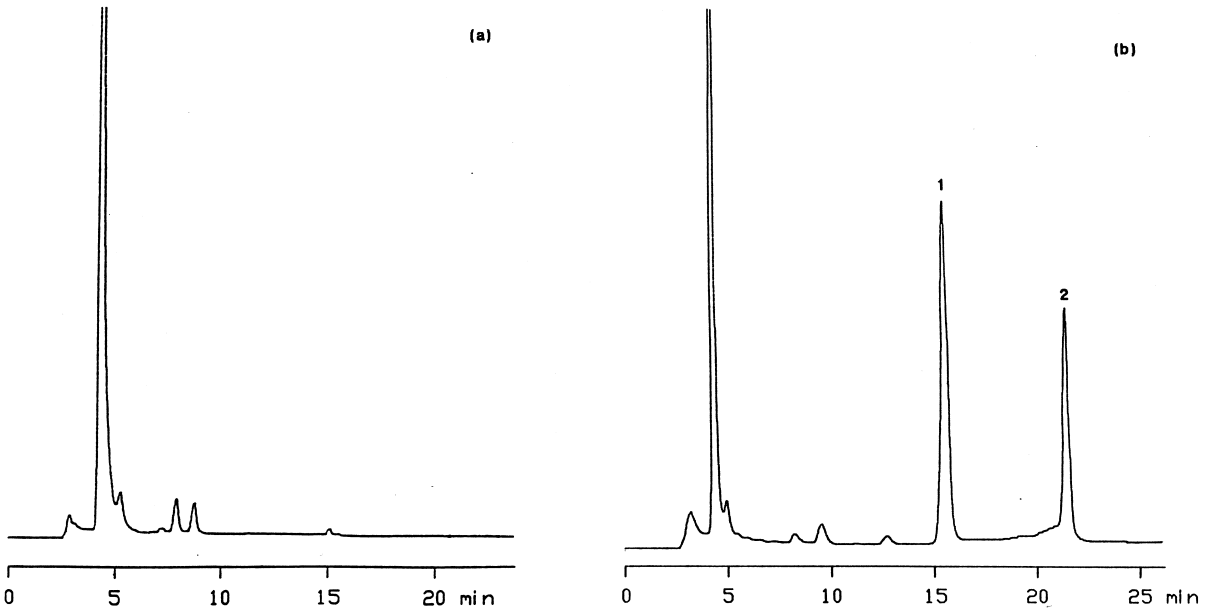

Fig. 2. (a) Chromatogram of a pool of blank turkey liver samples (n=6, control group). (b) Chromatogram of a blank turkey liver spiked with DOX (600 ng/g). Peaks: 1=DMCTC (I.S.) and 2=DOX. Conditions: column, PLRP-S 8 μm (250×4.6 mm I.D.) with a 5×3.0 mm PLRP-S guard cartridge; mobile phase, 0.01 *M* oxalic acid–acetonitrile–methanol gradient; flow-rate, 1 ml/min; fluorescence detection, λ_{ex}=406 nm, λ_{em}=515 nm.

respectively. These values are the result of repetitive analyses, obtained on the same day, and the within-day imprecision is expressed as the R.S.D. The between-day imprecision was 7.4% and 5.6% for liver and muscle, respectively (n=3). The maximum allowable tolerances for the imprecision (R.S.D.$_{max}$) for analyses carried out under repeatability conditions are one-half to two-thirds of the values calculated according to the Horwitz equation [20,21]. For liver tissue (MRL=300 ng/g) this R.S.D.$_{max}$ is maximal 9.6 to 12.8%. For muscle (MRL=100 ng/g) these values are 11.3 to 15.1%. The obtained R.S.D. for both within- and between-day imprecision are clearly below these R.S.D.$_{max}$ values.

3.2.3. Accuracy

The accuracy was defined as the closeness of agreement between the true value and the mean result of a series of experiments (n=6). It was determined by comparing the measured concentration to the spiked concentration. The concentrations used were equal to the MRL levels. The accuracy for these measured concentrations was +2.3% and +2.2% for liver and muscle, respectively and was within acceptable limits which are set at −20% to +10% for concentrations exceeding 10 μg/kg [21].

3.2.4. Linearity

The linearity of the assay was checked using spiked tissue with spike levels including the MRL. The concentrations examined were 0, 100, 200, 300, 400 and 500 ng/g in both muscle and liver tissue. The correlation coefficients r of the calibration curves were 0.9978 and 0.9994 for liver and muscle, respectively. Linear calibration curves were also obtained in spiked liver tissue between 0 and 1500 ng/g (six levels, r=0.9969).

3.2.5. Limit of detection

The limits of detection in matrix, using a signal-to-noise ratio of 3:1, were 1.2 ng/g and 1.0 ng/g in liver and muscle, respectively.

3.2.6. Stability

The stability of DOX in turkey liver during storage at −20°C was determined. Five liver samples

Table 2
Liver tissue concentrations of doxycycline in turkeys after storage for 22 days at −20°C

Turkey No.	Doxycycline concentration (ng/g)	
	Fresh	After 22 days at −20°C
1	158	173
2	233	239
3	200	214
4	349	341
5	314	306

were stored for 22 days at −20°C. The concentrations determined on the day of the collection of the liver samples and after storage at −20°C for 22 days are shown in Table 2. The concentrations after storage varied from −2.5% to +9.5% from the concentrations determined on the day of collection of the samples. A statistical paired Student's *t*-test ($\alpha = 0.05$) showed that the concentrations were not significantly different after storage, indicating good stability of DOX in liver samples.

3.3. Depletion kinetics and withdrawal time

The mean tissue concentrations±S.D. ($n=6$) in liver and muscle at 12, 36, 84, 180, 252 and 324 h after the last administration are given in Table 3. The correlation coefficient *r* between the residues in the liver and the muscle, taking all individual results into account was 0.9884.

The DOX tissue concentration versus time in the final depletion phase (beyond 36 h after the last administration) from both liver and muscle tissue is shown in Fig. 3. The terminal elimination half-life ($T_{1/2}$) of DOX was 77.7 and 78 h in muscle and liver, respectively, indicating that DOX was eliminated slowly, but with the same speed in both liver and muscle tissue. No information is available on residues of DOX in turkeys after medication via drinking water. Nevertheless, previous studies in other species have shown that DOX had a long elimination half-life in the final elimination phase, indicating the high affinity of DOX for tissues. Yoshimura et al. [22] administered DOX to laying hens via drinking water for seven consecutive days. DOX was detected in the albumin of the eggs until 24 days after the last administration and 26 days in yolk.

The linearity of the plot log concentration versus time indicates 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 [10]. Using this approach the withdrawal periods are determined at the time when the one-sided 95% upper tolerance limit with a 95% confidence level is below the MRL. For administration of 25 mg DOX·HCl/kg BW via drinking water for four successive days, a withdrawal time of 12 and 17 days might be necessary to assure that the concentrations in liver

Table 3
Tissue concentrations of doxycycline in turkeys after administration of 25 mg doxycycline·HCl/kg BW in the drinking water for four consecutive days

Tissue	Time after last dose (h)	Doxycycline concentration (ng/g) Mean±S.D. ($n=6$)
Liver	12	2369±695
	36	784±142
	84	369±114
	180	226±76
	252	252±65
	324	32±10
Muscle	12	2062±461
	36	642±65
	84	240±114
	180	151±62
	252	151±42
	324	26±8

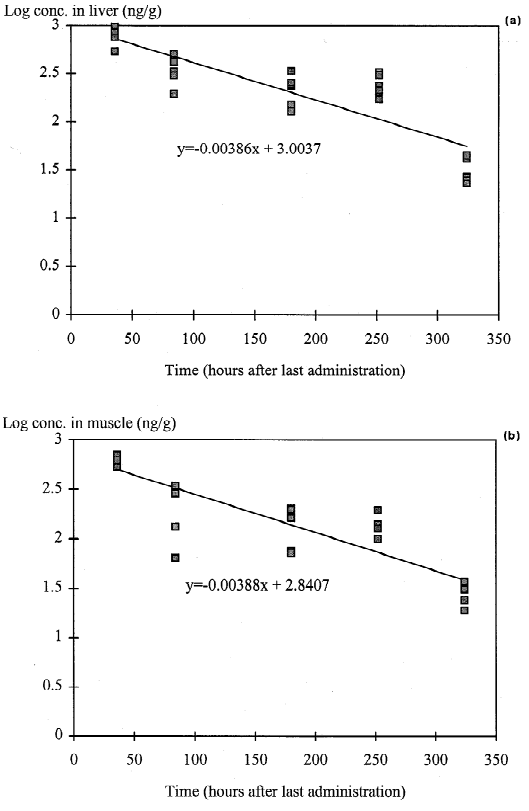

Fig. 3. Tissue depletion of DOX in turkey liver (a) and muscle (b) after administration of 25 mg DOX·HCl/kg BW via the drinking water for four days. The number of samples analysed at each sampling point is $n=6$.

and muscle tissue are below the MRLs of 300 ng/g in liver and 100 ng/g in muscle, respectively. These results clearly confirm the strong lipophilic characteristics of DOX resulting in a slow elimination. Anadón et al. [6] found that a withdrawal time of five days was necessary after oral administration of 20 mg DOX/kg BW in chickens for four successive days. This withdrawal time however, was established as the time point where the mean concentration of residues in all tissues was below the MRLs.

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