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TISSUE DISTRIBUTION AND RESIDUE DEPLETION OF OXYTETRACYCLINE IN THE RABBIT

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ABSTRACT : Oxytetracycline (OTC) is an antimicrobial drug widely used in the rabbit for treatment of intestinal and respiratory diseases sustained by *Pasteurella* spp, *Salmonella* spp. or E. Coli. This study was carried out to evaluate the distribution and residue depletion of oxytetracycline. The tissue distribution study was performed treating animals with a single dose of 80 mg of OTC *per* kg body weight (b.w.) in 2.7 kg rabbits. The residue studies were performed administering OTC *via* drinking water (680 ppm) or *via* pelleted food (1000 ppm). Real daily intake ranged from 50 to 80 mg *per* kg b.w. for 5 consecutive days. The drug tissue concentrations were determined by an HPLC method, with a quantification limit (LOQ) of 50 $mg.kg^{-1}$ and a detection limit of 5-6 $mg.kg^{-1}$according to considered tissue. The results of the study show that, in rabbits, OTC does not reach very high tissue concentrations following oral administration *via* medicated water or feed. Tissue residues fall below MRLs 72 and 48 hours following the withdrawal of medicated water and feed respectively. Tissue concentrations of OTC following administration *via* medicated water were greater than those recorded following administration *via* pellets : e.g. 200 to 700 *vs* <50 $mg.kg^{-1}$ in muscle or 200 to 600 *vs* 80 to 280 $mg.kg^{-1}$ in liver. One hour after the single dose administration, the concentration was 350-850 $mg.kg^{-1}$ in lung and twice of these values in kidneys.

RESUME : Distribution dans les tissus et vitesse d'élimination de l'oxytétracycline chez le lapin.
L'oxytétracycline (OTC) est un antibiotique largement employé chez le lapin pour le traitement des troubles respiratoires ou digestifs attribués à des *Pasteurelles*, des *Salmonelles* ou des colibacilles. Cette étude a été conduite de manière à déterminer la distribution de l'OTC dans les tissus de lapins de 2,7 kg après administration d'une dose unique de 80 $\mu g.kg^{-1}$. La vitesse d'élimination de l'antibiotique des tissus a été étudiée au cours des 42 heures suivant le retrait d'une médication par l'OTC soit via l'eau de boisson (680 ppm), soit *via* l'aliment (1000 ppm) appliquée pendant 5 jours chez des lapins de 2,7 kg également. La méthode de dosage de l'OTC (HPLC) avait un seuil de quantification de 50$\mu g.kg^{-1}$ et un seuil de détection de 5-6 $\mu g.kg^{-1}$ *selon les tissus*.
Une heure après une administration unique, les teneurs en OTC sont de 350 à 850 $\mu g.kg^{-1}$dans les *poumons et environ 2 fois plus élevées* dans les reins. A l'issue des 5 jours d'administration (ingestion réelle de 50 à 80 µg selon les individus) *la teneur en OTC dans les tissus* est plus élevée après administration via l'eau de boisson que via l'aliment : 200 à 700 contre < 50 $\mu g.kg^{-1}$ dans les muscles ou 200 à 600 contre 80 à 280 $\mu g.kg^{-1}$ dans le foie. La teneur en OTC résiduelle passe au dessous du seuil légal pour la consommation (MRLs) en 48 à 72 heures selon que l'antibiothérapie a été appliquée via l'aliment ou via l'eau de boisson

INTRODUCTION

In food-producing species, besides the safety of drugs, two main aspects must be guaranteed to ensure a proper pharmacological therapy. The first aspect concerns the demand to define dosing regimens suitable to achieve prefixed therapeutic objectives, with this aim experimental studies are carried out not only to satisfy clinical application requirements, but also to protect the environment and to reduce the cost of therapy using drug dosages useful to achieve effective tissue concentrations. The second aspect concerns the evaluation of the bio transformative pathways and the definition of the withdrawal times necessary to assure the absence of residues higher than MRLs in food-producing animals and so the healthiness of food destined to human consumption.

The above mentioned aspects justify the necessity to carry out experimental studies in the so-called *minor* species (equine, ovine-caprine, poultry [not-broiler], rabbits, fish [not-*salmonidae*]), for who available data is scarce, as stated in a CVMP Report (EMEA/CVMP/153a/97-FINAL). The breeding of some *minor* species and their use as food-stuff are related to cultural traditions and to geographical-economical aspects, which limit the spread on a wide scale. In Italy (as in other European countries), the production of foodstuffs from *minor* species has a primary importance only on a local scale, such as ovine-caprine and equine breeding in the southern regions or rabbit and eel breeding in the northern ones. Due to the fact that this production is rather limited and consequently not so important from an economical point of view, few studies have been published concerning the evaluation of tissue distribution and residue depletion of drugs.

Generally, tetracyclines are antibiotics which are safe and well tolerated, but adverse effects can be attributed to their irritant nature and disturbance of intestinal flora (REYNOLDS, 1996, PRESCOTT, 2000). These drugs have been used for many years against infections caused by susceptible micro-organisms because of their cheapness, broad antimicrobial activity, easy administration and good effectiveness. As known, the intestinal absorption of older tetracyclines (oxytetracycline, chlortetracycline and tetracycline) is usually reduced and this is partially due to the presence in feed of divalent cations (iron, calcium, magnesium) able to chelate tetracyclines (PERCY and BLACK, 1988; NIELSEN, 1997; RIVIERE

and SPOO, 1999; PRESCOTT, 2000). Tetracyclines are widely used in food-producing and companion animals in order to control systemic and local infections; due, in particular, to their well-known good distribution, they are useful in the treatment of mixed bacterial infections (PRESCOTT, 2000).

Oxytetracycline (OTC) is an old member of the tetracycline group effective against Gram positive and negative micro-organisms, *Chlamydia* spp., *Rickettsia* spp., *Mycoplasma* spp. and *Protozoa* spp.; it is widely used in the rabbit and, for this target species, many commercial preparations are available, that are indicated for treatment of intestinal and respiratory infections sustained by *Pasteurella* spp., *Salmonella* spp. and *E. coli*. Recently, due to high levels of resistance of intestinal micro-organisms, OTC is mainly used to control respiratory infections (BURGMANN, 2000, PRESCOTT, 2000).

Consequently, it has been considered useful to perform an experimental study on OTC tissue distribution (using a single administration by force-feeding) and residue depletion (using medicated water and feed) in rabbits in order to define its distributive capability in the most important tissues and to determine an appropriate withdrawal time following its oral administration.

MATERIALS AND METHODS

The whole experiment was performed using commercially bred rabbits (*Allevamento Perego, Comazzo, Milan, Italy*). The study was carried out on food-producing rabbits in order to define OTC tissue distribution and residue depletion in the normal breeding condition. The tissue distribution study was carried out administering OTC by force-feeding (single dose); and the residue study was conducted by administering OTC dissolved in drinking water or pellets that had been specially prepared (Dottori Piccioni, s.n.c., Milan, Italy). In the trial involving medicated feed, the pellets were prepared using the same ingredients contained in the commercial feed given to the entire sample of animals before treatment and to the control sample animals during the experimental period. Before the administration of medicated feed, the level of OTC concentration was determined in the laboratory by HPLC (CAPOLONGO *et al.*, 2000) : the mean concentration was found to be 967 ± 65 $mg.kg^{-1}$ feed. The calcium content of commercial and medicated feed was about 0.7%.

Tissue distribution study

Animals :

29 hybrid rabbits were used, both sexes, mean weight 2.7 kg, clinically healthy. The animals were caged individually (in accordance with the requirements set out in *Gazzetta Ufficiale della Repubblica Italiana*, no. 40, 18.02.1992) and housed for an acclimatisation period of ten days before the experiment. During this period, food and water were given *ad libitum*. Eight animals out of 29 were not fed for 12 hours before the treatment in order to verify the influence of food on the intestinal absorption of OTC.

Treatment :

The subjects were treated at the dose of 80 mg/kg b.w. by individual force-feeding using a commercial preparation (*Ossitetraciclina 20% liquida - TREI S.p.A.*, Modena, Italy). The drug was administered through a mouth gag prepared from the plastic case of a 5 ml syringe and using a urinary catheter (Rusch Nelaton 40 CH.14).

Sacrifice and Sample collection :

The fed animals (21) were divided *ad random* into 3 groups of 5 subjects (3 males and 2 females) and 2 groups of 3 subjects (2 males and 1 female); the groups were sacrificed 1, 3, 6, 9 and 12 hours after the treatment, while the fasted rabbits were divided *ad random* into two groups of 4 animals and sacrificed 1 and 3 hours post-treatment. The animals were euthanized in a CO_2 chamber in accordance with the requirements set out in Recommendations for Euthanasia of Experimental Animals (*Commission of the European Communities*, Final Report, May 1993). From each animal were collected samples of muscle, liver, kidney, and lung. The samples were stored at – 80°C pending assay.

Residue depletion study

Animals :

48 hybrid rabbits were used, both sexes, mean weight 2.7 kg, and clinically healthy. The animals were caged individually (in accordance with the requirements set out in *Gazzetta Ufficiale della Repubblica Italiana*, no. 40, 18.02.1992) and housed for an acclimatisation period of ten days before the experiment. During this period, feed and water were given *ad libitum* and feed and water consumption registered daily.

Treatment:

The first 24 animals were fed with commercial pellets not containing any active ingredient able to interfere with OTC titration, and treated with medicated water containing OTC at the concentration of 680 ppm for five days. The medicated water was prepared using a commercial OTC product (*Ossitetraciclina 20% liquida – TREI S.p.A.*, Modena, Italy). The concentration of the medicated water was calculated according to the daily water consumption in order to obtain a daily intake of about 80 $mg.kg^{-1}$ of OTC. The water solutions were prepared daily and administered through individual bottles to the rabbits.

The individual water consumption, and consequently the assumption of the drug, were recorded daily. The other 24 rabbits were fed for five consecutive days with pellets containing 1000 ppm of OTC. The antibiotic concentration in the pellets was calculated on the basis of the daily food intake in order to obtain the daily assumption of a dose of about 80 $mg.kg^{-1}$ of OTC. The food consumption was calculated daily.

Sacrifice and Sample collection:

Starting from the end of the administrations, the two groups of animals were subdivided *ad random* in groups of six (3 males and 3 females) and sacrificed after 0, 24, 48 and 72 hours. The animals were euthanized in a CO_2 chamber in accordance with the requirements set out in Recommendations for Euthanasia of Experimental Animals (*Commission of the European Communities*, Final Report, May 1993). From each animal were collected samples of muscle, liver and kidney that were stored at – 80°C pending assay.

Method of analyses

The analyses were carried out by liquid chromatography (HPLC) adapting a specific method developed by the Institute of Veterinary Pathology and Hygiene of the University of Padova (CAPOLONGO *et al.*, 2000). The tissue samples (1 g) were homogenised with 7.5 ml of extraction buffer (A: citric acid 0.1 M; B: sodium hydrogen phosphate dihydrate 0.2 M [A 62%: B 38%]) and 7.5 ml of methanol. After centrifugation (10000 *g* for 10 min at 4°C), the liquid phase was cleaned up using a mini SPE column prepared using as stationary phase the "Chelating Sepharose" (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The elution was carried out using 0.8 ml of McIlvane buffer, which was increased to 1 ml using further McIlvane buffer. The samples were immediately assayed.

OTC was analysed by HPLC (Perkin Elmer: Pump LC 250; UV Detector LC 90; Autosampler ISS 200; Turbochrom software). The chromatographic column was a C18 *Luna* 5 µm, 150 x 4.6 mm (Phenomenex, Torrance, USA). The chromatographic conditions were: mobile phase oxalic acid 0.01 M and acetonitrile (85:15); flow rate 1 $ml.min^{-1}$ and UV detection 355 nm.

The limit of quantification (LOQ) was 50 $\mu g.kg^{-1}$ in all the matrixes; the limits of detection (LOD) were 5.13, 6.12, 5.41 and 5.87 $\mu g.kg^{-1}$ for muscle, liver, kidney and lung respectively. The method was validated intra-laboratory and found to be satisfactory for specificity, reproducibility, repeatability and accuracy. The mean recoveries were 84.36%, 71.7%, 70.26% and 80.56% for muscle, liver, kidney and lung respectively. The method was also validated for 4-epi-OTC (supplied by Acros, USA), a metabolite that could occur during the extraction phase. In the samples analysed, the temperature was kept below 5°C during all the extraction steps and no 4-epi-OTC was detected.

Statistical Analysis

Considering the great individual variability within the experimental groups and the consequent dispersion of the data, the statistical analysis was carried out with the distribution free Kruskal–Wallis (KW) test, followed by a post KW test for multiple comparisons ($P \leq 0.05$) (SIEGEL and CASTELLAN, 1992).

RESULTS

The experimental data obtained following individual administration by force-feeding are reported in Table 1. Peak concentrations were achieved about 1 hour after treatment in kidney, liver and lung, while in muscle the concentrations recorded at the different time-points were similar and significantly lower than the ones observed in the other tissues. The kinetic profile of tissue concentrations shows a constant decrease (except in muscle) from the 1st hour until the 6th hour post-treatment and then values remain almost constant until the 12th hour. The statistical analysis

Table 1 : OTC Concentrations ($\mu g.kg^{-1}$) after force-feeding administration in fed animals at 80 $mg.kg^{-1}$

Sacrifice time	Animal number	Liver	Kidney	Muscle	Lung
1h	1	828	1666	207	865
1h	2	276	498	55	762
1h	3	324	1364	177	583
1h	4	2490	1720	75	795
1h	5	260	1462	<LOQ*	348
3h	6	280	886	293	204
3h	7	260	1118	126	327
3h	8	230	569	93	212
3h	9	290	1099	134	249
3h	10	1300	356	<LOQ	899
6h	11	149	318	85	<LOQ
6h	12	162	323	<LOQ	154
6h	13	120	300	<LOQ	67
6h	14	90	241	56	<LOQ
6h	15	108	614	131	<LOQ
9h	16	193	580	61	52
9h	17	99	440	<LOQ	ND**
9h	18	360	584	209	ND
12h	19	200	752	140	141
12h	20	110	455	240	219
12h	21	120	407	95	260

* <LOQ=Residues below the LOQ (50 $\mu g.kg^{-1}$) were considered as half LOQ in statistical analyses

** N.D.=Not detected, considered as half LOD (2.56, 3.06, 2.70 and 2.93 $\mu g.kg^{-1}$ in muscle, liver, kidney and lung, respectively)

Table 2 : OTC Concentrations ($\mu g.kg^{-1}$) after force-feeding administration in fasted animals at 80 $mg.kg^{-1}$

Sacrifice time	Animal number	Liver	Kidney	Muscle	Lung
1h	22	300	963	ND**	1340
1h	23	1050	1674	113	1575
1h	24	209	431	ND	166
1h	25	207	356	ND	228
3h	26	545	1410	157	1228
3h	27	256	530	ND	ND
3h	28	ND	160	ND	ND
3h	29	<LOQ*	300	ND	ND

* <LOQ = Residues below the LOQ (50 $\mu g.kg^{-1}$)
** N.D. = Not detected

(Kruskal-Wallis test) shows significant differences ($P<0.05$) in the antibiotic concentrations in kidney and lung collected from the animals belonging to the groups sacrificed 1, 3 and 6 hours post-treatment, while in liver significant differences ($P<0.05$) were observed only in the groups sacrificed 3 and 6 hours following treatment. In muscle, no significant differences were observed between the different groups.

The experimental data recorded after individual single administration by force-feeding in fasted animals are reported in Table 2 and show the achievement of peak concentrations about 1 hour after treatment in kidney, liver and lung. In muscle, OTC concentrations were below the LOQ 1 and 3 hours post-treatment in three animals out of four; only in one rabbit sacrificed at the 1st hour and in one at the 3rd it has been possible to quantify OTC concentrations (110 $\mu g.kg^{-1}$ and 160 $\mu g.kg^{-1}$, respectively). The recorded concentrations were lower than those observed in fed animals. It should be stressed that, during necropsy, a relevant –relative, large?- quantity of food was still found in the gastrointestinal tract and, for this reason, further studies are necessary to be able to draw any conclusions regarding the effect of food on OTC absorption.

With regard to the continuous administration (5 days) of medicated water (680 ppm), the higher tissue concentrations are recorded at the suspension of treatment, as reported in Table 3. The decrease of concentrations was rapid, reaching values lower than the LOQ (50 $\mu g.kg^{-1}$) at the 72nd hour. Concentrations near the MRLs defined for OTC (liver 300 $\mu g.kg^{-1}$, kidney 600 $\mu g.kg^{-1}$ and muscle 100 $\mu g.kg^{-1}$) (Gazzetta Ufficiale delle Comunità Europee, 1999) were observed for all the tissue matrixes at withdrawal times between 24 and 48 hours.

Table 3 : OTC Concentrations ($\mu g.kg^{-1}$) after continuous administration (5 days) of medicated water (680 ppm)

Withdrawal time	Animal number	Liver	Kidney	Muscle	Daily OTC intake ($mg.kg^{-1}$)
0h	30	436	1358	690	59.46
0h	31	284	717	500	60.23
0h	32	561	1184	729	87.10
0h	33	196	614	297	58.36
0h	34	250	348	178	48.73
0h	35	179	680	177	56.79
24h	36	<LOQ*	218	282	54.52
24h	37	<LOQ	261	129	48.59
24h	38	197	436	322	75.90
24h	39	<LOQ	229	142	82.59
24h	40	<LOQ	85	<LOQ	63.28
24h	41	<LOQ	202	<LOQ	75.56
48h	42	ND**	ND	126	59.36
48h	43	<LOQ	85	146	72.67
48h	44	261	375	53	90.86
48h	45	ND	ND	ND	61.33
48h	46	ND	ND	ND	55.85
48h	47	ND	ND	ND	75.43
72h	48	ND	<LOQ	ND	49.45
72h	49	ND	57	ND	59.76
72h	50	ND	ND	ND	56.76
72h	51	ND	ND	ND	70.82
72h	52	ND	ND	ND	56.60
72h	53	ND	ND	ND	60.44

* <LOQ=Residues below the LOQ (50 $\mu g.kg^{-1}$) were considered as half LOQ in statistical analyses
** N.D.=Not detected, considered as half LOD (2.56, 3.06, 2.70 and 2.93 $\mu g.kg^{-1}$ in muscle, liver, kidney and lung, respectively)

Many individual variations were also observed in this experiment; these are partially attributable to the different individual drug intakes, as reported in the last column of Table 3 where individual OTC intakes are recorded.

The statistical analysis (Kruskal-Wallis test) shows significant differences ($P<0.05$) among the sacrifice times (0, 24, 48 and 72 hours post-treatment) in all the matrixes collected.

Concerning the continuous administration (5 days) with medicated pellets (1000 ppm), the tissue concentrations recorded at the suspension of treatment, as

Table 4: OTC Concentrations (μg.kg^{-1}) after continuous administration (5 days) with medicated pellets (1000 ppm)

Withdrawal time	Animal	Liver	Kidney	Muscle	Daily OTC intake (mg.kg^{-1})
0h	54	276	402	<LOQ	63.64
0h	55	92	561	ND	75.50
0h	56	83	433	ND	60.47
0h	57	110	600	ND	61.32
0h	58	160	480	ND	58.98
0h	59	80	630	<LOQ	49.98
24h	60	<LOQ*	135	ND	60.00
24h	61	ND**	106	ND	61.78
24h	62	<LOQ	159	ND	62.55
24h	63	ND	180	ND	62.01
24h	64	<LOQ	110	ND	60.23
24h	65	ND	210	ND	59.88
48h	66	ND	ND	ND	47.50
48h	67	ND	<LOQ	ND	60.64
48h	68	ND	ND	ND	64.04
48h	69	ND	<LOQ	ND	60.65
48h	70	ND	ND	ND	63.00
48h	71	ND	ND	ND	58.88
72h	72	ND	ND	ND	64.42
72h	73	ND	<LOQ	ND	56.34
72h	74	ND	ND	ND	57.24
72h	75	ND	ND	ND	59.63
72h	76	ND	<LOQ	ND	60.11
72h	77	ND	ND	ND	61.02

* <LOQ=Residues below the LOQ (50 μg.kg^{-1}) were considered as half LOQ in statistical analyses

** N.D.=Not detected, considered as half LOD (2.56, 3.06, 2.70 and 2.93 μg.kg^{-1} in muscle, liver, kidney and lung, respectively)

reported in Table 4, were above the LOQ in the case of kidney and liver, but below in the case of muscle. At the second time-point (24 hours), it was possible to quantify residues in all animals only in kidney. In liver, only three animals out of six had detectable residues, while in muscle no residues were detected in any of the samples. At 48 and 72 hours of withdrawal time residues were not detectable in liver and muscle, while in kidney only two samples *per* time-point presented residues, but at concentrations below the LOQ (50 μg.kg^{-1}).

The statistical analysis (Kruskal-Wallis test) shows significant differences ($P<0.05$) in kidney and liver among the groups sacrificed at 0, 24 and 48 hours after treatment. In muscle, the Kruskal-Wallis test shows a significant difference only between group 0 and 24, but it has to be underlined that residues were already below the LOQ at the first time-point (0 hours).

The mean doses assumed by the animals treated with medicated water or feed (64.1 ± 11.8 and 60.4 ± 5.1 mg.kg^{-1} respectively) were significantly lower than we had forecast (80 mg.kg^{-1}). This is probably due to the less palatable taste of medicated water and feed: the mean intake of water and feed recorded for the animals before treatment were 112.2 ± 16 ml.kg^{-1} and 75 ± 10 g.kg^{-1}, respectively, *versus* 94 ± 17 ml.kg^{-1} and 60 ± 5 mg.kg^{-1}, recorded during the treatment period.

DISCUSSION

OTC tissue concentrations attained following oral administration were found to be lower than expected and influenced by the method of administration. With regard to the effect of gastric repletion on drug absorption, the pilot trial carried out using fasted animals did not provide any conclusive results due to the fact that the trial period of 12 hours was not sufficient to achieve gastric emptying in the animals and, probably, also due to the well-known caecotrophy habit of rabbits.

The antibiotic tissue concentrations recorded following single administration by force-feeding in fed animals or by medicated water and feed were partially in accordance with our expectations. The levels observed at the time of first sacrifice (1 hour) after the single dose of 80 mg.kg^{-1} by force-feeding were higher than those recorded at the first time point (*zero* hour) following treatment by medicated water or feed. These results are due to the difference between the mean doses assumed by force-feeding or diet (80 mg.kg^{-1} *versus* about 64 and 60 mg.kg^{-1} for medicated water and feed, respectively) and between administration of the drug as bolus or by continuous intake during the whole day *via* a medicated diet (water or feed).

The results of the trial involving administering OTC by force-feeding show that the antibiotic diffuses rapidly (1 hour post-administration) in all the tissues analysed.

The differences between the depletion results recorded in trials involving medicated water and feed (higher and more persistent OTC tissue levels were recorded following administration *via* water) were also in line with expectations and can be attributable to the

generally better absorption of drug administered *via* drinking water than *via* feed.

The analytical method used for the detection of OTC in the different matrixes resulted specific and accurate and also able to detect the 4-epi-OTC metabolite. The extraction procedure is easily applicable, but not very rapidly as only a few samples can be processed in a day.

However, the results of the trials enable us to conclude that the withdrawal time normally adopted for OTC commercial preparations given to rabbits (at least 5 days) may be reduced to 3 days in the case of OTC administration *via* medicated water or feed whilst still respecting established MRLs and the requirements for the production of safe foodstuffs for commercial rabbitry.

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