Abstract

Tissue distribution and depletion of chlortetracycline hydrochloride was studied after in-feed administration to hybrid Comb broilers under controlled experimental field conditions. The pharmaceutical product Vitachlor 100 with a content of 10% of chlortetracycline hydrochloride was mixed in the feed at 4.0 kg/tone of feed in order to be given to broilers at the recommended therapeutic dose of 20 mg/kg b.w./day/5 days. Eight sampling points were performed of six broilers each at 24 h, 72 h and 120 h during medication and on 24 h, 48 h, 72 h, 120 h and 240 h after medication. Muscle, liver and kidney tissues were collected from each broiler and stored at -45°C until the analysis. Chlortetracycline was extracted by McIlvaine/EDTA buffer pH 4.0 on the presence of TCA 20% solution and the cleanup and isolation of the chlortetracycline was performed on a SPE IST-C18 minicolumn. The detection, identification and quantification of the chlortetracycline hydrochloride residues were performed by high performance liquid chromatography and a Photo Diode Array detection-HPLC-PDA. The concentration of chlortetracycline hydrochloride residues in the target tissues was not detectable 24 h after medication. The highest chlortetracycline concentrations were determined in kidney (2841.5 μg/kg) and in liver (280.5 μg/kg) during the medication period and just in kidney 24 h after medication (19.25 μg/kg). The mean concentrations of chlortetracycline in muscle tissue on the first day after medication was 5.15 μg/kg e.g. quite less than the legislative MRL in the European Union, which means that broiler’s muscle tissue is safe for the consumer. A Withdrawal Period of 24 hours for the Maximum Residue Limit (MRL) of 100 μg/kg chlortetracycline in muscle tissue is recommended.

Keywords: chlortetracycline, residues, broilers, hplc, withdrawal period

Introduction

The term «antibiotic» is normally reserved for a very diverse range of compounds, both natural and semi-synthetic, that possess antibacterial activity. They fall into five classes: penicillins, tetracyclines, macrolides, aminoglycosides and amphenicols. Strictly speaking, sulfonamides, nitroimidazoles, nitrofurans and quinolones are not antibiotics, being synthetic. These compounds have been used for many years in food producing animals for the treatment of disease, prophylactically as well as to promote growth (Kennedy et al. 1998). TCs are a wide range active antibacterials with bacteriostatic and bacteriocide properties against + Gram and – Gram bacteria. They also act against ricketsiosis, mycoplasms, Chlamydia and protozoa. It is used per os or parenterically and shows rapid absorption and tissue distribution. They are actively transported into cells of susceptible bacteria where they exert a bacteriostatic effect by inhibiting protein biosynthesis after binding to the 30S ribosomal subparticle of the bacterial ribosom inhibiting the production of protein substances e.g. enzymes (Oka 1995). TCs are rapidly absorbed through the various routes of administration and are widely distributed in the body with highest levels in kidney, liver, bones and dentine. They undergo minimal or no metabolism and they are excreted in urine and feaces either unchanged or in a microbiologically inactive form. Although there are differences between them (OTC, TC, CTC) in percentage urinary and fecal excretion, these differences are not substantial. It should be noted that given the polarity of these substances they are not detectable in fat to any great extend and for their complete recovery, the 4-epimers have to be determined. These compounds occur in samples and are formed during sample preparation. The 4-epimers are in equilibrium with the parent compound and therefore the marker
residue is the sum of the parent drug and its 4-epimers (EMEA 1995a). More specifically, CTC is produced by fermentation during which tetracycline and demeclocycline are produced as well as other compounds (called impurities) such as: iso-chlortetracycline in alkaline environment, 4-epi-chlortetracycline in acid environment, 4-epi-tetracycline and 4-epi-demeclocycline following the epimerization of the parent compound (Clarke’s 1986). The chemical structure of chlortetracycline can be seen in figure 1 and the molecular structure of the hydrochloric form is $C_{22}H_{23}ClN_2O_8\cdot HCl$ with relative molecular weight $mw = 515.3$.

According to the EU legislation and EMEA (The European Agency for the Evaluation of Medicinal Products), CTC has been included in Annex I of the Regulation (EEC) No. 2377/90 with the proposed Maximum Residue Limits (MRLs) included in table 1 and with target tissues kidney, liver, muscle, milk and eggs. The same MRL values valid for chickens with target tissues muscle, liver and kidney (EMEA 1995a, Reg. 508/1999/EC). Taking into account EMEA’s values our intention was focused on CTC hydrochloride residue determination in relation to the elimination time from muscle, liver and kidney as well as on it’s the excretion validation until concentration levels of less than the proposed MRL so that the final product coming to the market to be safe for the consumer.

Materials and Methods

CTC determination in muscle, liver and kidney of broilers was performed by the method of Tyrpenou (1995) with some modifications and validated for the determination of CTC hydrochloride residues in muscle, liver and kidney of broilers. Feed analysis, with and without the addition of the premix Vitachlor 100 as well as the premix itself was performed by hydrochloric acid extraction as it is suggested in the literature (Holland et al. 1991; Houglum et al. 1997) and in the European Pharmacopoeia (EP).

Reagents and chemicals

CTC hydrochloride CRS (Certified Reference Standard), was provided by the Council of Europe, European Pharmacopoeia, BP 907- F67029 Strasbourg CEDEX 1 with a purity of 91.7% according to the certificate of analysis. HPLC organic solvents were used and the rest of the reagents were of analytical purity grade. The Solid Phase Extraction C$_{18}$ mini-columns were of 500 mg and coming from IST Company. CTC hydrochloride stock solution of 1000 μg/mL as well as the intermediate solutions of 100 and 10 μg/mL were prepared in HPLC methanol. Working standard solutions of 0.25 μg/mL, 0.50 μg/mL, 1.0 μg/mL, 2.0 μg/mL, 4.0 μg/mL, 6.0 μg/mL, 8.0 μg/mL and 10.0 μg/mL were prepared in HPLC water. The quantities injected into the chromatograph with a volume of 100 μL gave 0 ng, 25 ng, 50 ng, 100 ng, 200 ng, 400 ng, 600 ng, 800 ng and 1000 ng, respectively (external standard curve). CTC stock solutions were stored at -45°C for 6 months while working standard solutions were always prepared with each new batch of samples.

High Performance Liquid Chromatographic system

The HPLC analytical system Alliance 2690XE Separation module, Revision 1.21 (Waters, USA) was used for the determination of CTC hydrochloride residues coupled with a UV/vis Photodiode Array (PDA) model M996. The chromatographic column used was Zorbax SB-C$_{18}$, 250 mm x 4.6 mm id, 5 μm, HP (Germany), and the control of the LC system, data acquisition and peak integration were performed by the software Millennium32 Chromatography Manager (rev.1.21) (Waters USA).

Chromatographic conditions

The chromatographic conditions used were:

Analytical column: Zorbax SB-C$_{18}$, 5 μm (250 mm x 4.6 mm)
Mobile phase : (A) acetonitrile & (B) trifluoroacetic acid 0.1% (v/v), pH 2.0
Mobile phase flow : 0.8 mL/min, and gradient programme:

<table>
<thead>
<tr>
<th>Time</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>30.0</td>
<td>70.0</td>
</tr>
<tr>
<td>2.00</td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td>10.0</td>
<td>70.0</td>
<td>30.0</td>
</tr>
<tr>
<td>12.0</td>
<td>30.0</td>
<td>70.0</td>
</tr>
</tbody>
</table>
Equilbr. time : 3 min

Chromatographic run : 10 min
Column temperature : 35°C
Autosampler’s temp. : 15°C
Degassing system : normal
Wavelength : $\lambda_{\text{max}} \sim 365$ nm
Injection volume : 100 µL

**Biological experiment**

The first part of this study, concerning the biological experiments as well as sample collection and conservation, was performed in the premises of the Agricultural University of Athens and the whole procedure was according to the principles and the EU legislation. Before the biological experiment animals were kept for 6 days acclimatization period to the new conditions. Broilers’ veterinary health control, body weight as well as mean temperature and humidity registration were performed and one day before starting the experiment broilers were kept unfed. This experiment was started on 30th June 2003 and finalized on 22nd July 2003 e.g. it was concluded in a period of 22 days during which the mean environmental temperature was $22-28 \pm 0.8 \ ^\circ C$ and just during a few hours of the day a temperature of $35 \ ^\circ C$ was registered with a mean relative humidity of $30-40 \pm 5\%$. Fifty four (48 + 6 control) healthy broiler hybrid Comb of 32 days and of 1000 - 1100 g mean weight were used by ensuring that no pharmaceutical treatment has been used during their life except the necessary vaccines, (Gumboro, Marek, Mew Castle disease, Infectious bronchitis). In table 2, broilers’ mean body weight increase can be seen during the biological experiment.

Broilers were transferred to the premises of the Agricultural University of Athens during the night (10:00 pm) and separated in cages with 6 chicks per cage. A standard feed was given to the broilers (mean feed consumption 100-120 g per day) and water *ad libidum* and then the medicated feed with the pharmaceutical product Vitachlor 100 (batch number: 742301) at the therapeutic dose of 20-40 mg/kg b.w. and for 5 consecutive days. At the same time 6 broilers were fed the same feed without Vitachlor 100 (control broilers). The synthesis of the feeding stuff used in this study in kg/ton was: corn 85 kg, soft maize 535 kg, soya 48% 275 kg, soya oil 36 kg, nitrophos 22% 12 kg, vegetable oil 25 kg, marble powder 17 kg and equilibrator 15 kg. Before starting the experiment a chromatographic analysis of the commercial feed was performed to check the absence of CTC (6 samples) as well as of the medicated feed (12 samples). CTC was not detected in all samples of the commercial feed used and in the medicated feed it was found a homogenous CTC distribution in all samples examined (all samples contained the same CTC quantity).

Six (6) samples of 6 broilers each were taken at 24 h, 72 h and 120 h during medication and at 24 h, 48 h, 72 h, 120 h and 240 h after the last medication day. Following the registration of the broilers’ weight the target tissue samples (muscle, liver, kidney) were taken from each chicken in total 3 x 54 =162 samples and they were kept at $-45\ ^\circ C$ until the analysis. Also, the same samples were taken from each control chicken (control samples).

**Results and Discussion**

As we can see in figure 2, CTC concentrations were increased from first to third medication day and then a slight decrease was observed until the fifth medication day which indicates the gradual increase and decrease of CTC. The interesting point is that the first day after medication CTC was disappeared rapidly from the tissues, especially from muscle and liver, showing higher concentrations only in kidney.

The statistical programme WT1.1 (Withdrawal Time Calculation Program), which is suggested by the EMEA (CVMP 1996, Hekman 2000) calculates withdrawal period using specific experimental data, such as the number of the groups used from 3-7 and the number of the animals per group from 2-15. In our case, the analytical data, starting from the first day’s group after medication, showed that CTC hydrochloride residues were disappeared directly after medication and the analyses performed until the third day after medication showed complete absence of CTC residues (Not Detected). According to the literature withdrawal period is determined when the tolerance limit of the analyte concentration is exactly at the MRL or lower. When the calculated period is a part of the period of 24 h then the next day is calculated as withdrawal period. According to our experimental data for the broilers and for a Maximum Residue Limit of 100 µg/kg as Withdrawal period could be safely set a period of 24 h after the last medication day. In the literature, CTC half-life in blood plasma is 6 h. Taking into account this information and the results of our study it is impossible this parameter to be calculated by using the equation $t_{\text{1/2}} = 0.693/k$ (where 0.693 = Ln 2 and k is the slope of the bi-exponencial curve), because there no data from the days after the last dosing.

During the application of the analytical method on broiler’s muscle and at the wavelength of 365 nm, it was observed that a small stable in height peak was interfering and obstructed CTC identification because of it was
eluted at the same time (Rs=1). We Gradient elution was then selected and after equilibration and stabilization of distribution procedure, CTC was separated from the interfering peak (Rs=1). The retention time of CTC was \( R_t = 5.88 \pm 0.0218 \text{ min} \) with a relative standard deviation \( RSD = 0.28\% \), a parameter which characterizes pump flow stability and precision. Ten (10) injections were performed for the determination of the retention time in different days (between-day injections) and gave \( RSD \leq 5\% \), which is in compliance with the criteria for the accuracy of the results. Concerning the spectrum of CTC two maximum were observed at the wavelength of \( \lambda_{\text{max}} \sim 365 \text{ nm} \) one at 266 nm and another at 368 nm. CTC confirmation was performed by comparing the spectrum of the pure CTC standard to that of the sample based on the ability of PDA.

In general, OTC and TC residues are detected and identified more easily with HPLC analysis than that of CTC residues with recoveries (R%) in muscle 10-20% higher than that of CTC. On the contrary, CTC can be detected at concentrations 3 times lower than that of OTC and TC by inhibiting microbiological methods. In addition, because TCs are eliminated very fast from fat tissue European Commission (EC) decided that this tissue not to be included in the target tissues and no MRL to be specified. Also, EC concluded that TCs residues could easily be detected in animal tissues at levels between 100-600 μg/kg depending on tissue-tetracycline combination (MacNeil et al. 1996). Also, it is well known from the literature that TCs, although they pose the same physicochemical properties, the most active is CTC. They are amphoteric chemical compounds with characteristic pKₐ values, with UV spectrum which has an intense absorption between 260 and 360 nm in neutral and acid environment. They are soluble in acids, bases, alcohols and in polar organic solvents and they can be extracted with organic solvents such as n-butanol and ethyl acetate. They fluoresce in the presence of metal ions or in basic conditions. Its stability is low in strong acid or basic conditions resulting in the reverse formation of the epimers (4-epi-tetracyclines) in pH 3.0 (Oka et al. 1995). Epimers which are formed under abnormal conditions of temperature, pH and humidity (reversible epimerization) show very weak antibacterial action. Because these products have shown a slight toxicity, it is very important to be determined during the analysis (Lotfi et al. 2000). It is mentioned that CTC epimers in muscle tissue of broilers is impossible to be determined with accuracy because of low resolution (Mulders et al. 1989). They form chelate compounds (complexes) with metal ions, they bound to proteins and to silanols on the stationary phase of the chromatographic system causing analytical problems. Because of these properties since 1985 this problem has been overcome by using ethylene-diamine-tetraacetic acid (EDTA) and oxalic acid for their extraction, cleanup and isolation (Oka et al. 1995). Also, for their extraction from tissues it is suggested McIlvaine at pH 4.0 to be used but it should be cold, the mechanical homogenization to be less preferably to use vortex and sonication at 50 ± 60 kHz as well as centrifugation under cold conditions e.g. at 10°C (Kühne et al. 2001a). TCs they excreted in the environment as parent compounds as well as metabolites. The main metabolic products are formed because of their low stability in the water, pH, metal ions and light and some of them (5a,6-anhydro-tetracycline and 5a,6-anhydrochlortetracycline) pose antibacterial action against resistant to TCs bacteria (Halling-Sørensen 2002). It should be noted that TCs are unstable and their concentrations are getting lower at temperatures of 100°C - 133°C and CTC is destroyed at a percentage of 90-100% (Kühne et al. 2001b).

According to EMEA statistical analysis the analysis of variance needs data which should be between them independent. Normally, all the data of a residue study for withdrawal period calculation follow this regulation because they are coming from independent animals. In cases of double and triple samples the mean is calculated but standard error impact to be final results, in most of the cases, is very small in comparison to the variation between animals. When all or most of one slaughter day data show much difference then these figures could result to the rejection of this point. We should keep in mind that 3 time points are necessary for the regression analysis to be valid. In theory, a minimum number of 3 animals per 3 slaughter points at least is needed for the residue logarithmic curve (logₑ-linear phase of the terminal elimination of residues). Suggestions on the number of animals to be used in a residue study is included in volume VI of the Rules Governing Medicinal Products in the EC, Part IV, 1991 where, accordingly to the animal species, 4-10 animals per time point is permitted.

Also, figures usually cited as they have been measured namely without correction for recovery although this correction is performed many times by using several supporting recovery data (EMEA, 1995b). Relevant results from another old study (Wells, 1996) in broilers with 220 mg/kg CTC in feed, on zero day after medication residues have been found at concentrations of 0.66 and 0.71 mg/kg in liver and 0.42 and 0.75 mg/kg in kidney, respectively (broilers were older than the broilers of our study). All that mentioned before, such as CTC particularities during liquid chromatographic analysis, its properties in relation to the tissue, the concentration levels found in older studies as well the recommendations of the EMEA, they have been taken into account in the standardization of the CTC method, in the planning out of the biological experiments and in the statistical analysis.
Conclusions

The results of this study, based on the efficiency of the analytical method used showed that CTC hydrochloride residues are eliminated rapidly from broilers’ tissues (muscle, liver, and kidney) and more specifically:

The concentrations of CTC hydrochloride residues in muscle after the last medication day were found to be very low in relation to the Limit of Quantification (LOQ) of the method used and to the MRL 100 μg/kg.

The concentrations of CTC hydrochloride residues in liver after the last medication day were found to be very low too in relation to the Limit of Quantification (LOQ) of the method used and to the MRL 300 μg/kg.

The concentrations of CTC hydrochloride residues in kidney after the last medication day were found to be higher but very low too in relation to the Limit of Quantification (LOQ) of the method used and to the MRL 600 μg/kg.

Half-Life of CTC hydrochloride determination was not able to be calculated because concentration levels of CTC after the last medication day were quite below from the lower limit of detection of CTC by the PDA detection at 365 nm.

A Withdrawal period of 24 h, after the last medication day and for MRL 100 μg/kg CTC in broiler’s muscle tissue, is suggested. According to the suggested withdrawal period it is clear that CTC hydrochloride residues concentrations in muscle tissue well as in the other target tissues after in feed administration to broilers are at levels below the MRL 100 μg/kg which means that the final product in safe for the consumer.

Literature


CVMP NOTE OF GUIDANCE. (1996) Considerations on establishing withdrawal periods, Food and Drug Administration, U.S.A.


HEKMAN, P. (2000) BRD Agency for the Registration of Veterinary Medicinal Products, The Netherlands


Figure 1. Chemical structure of chlortetracycline.

Figure 2. Histogram of the elimination to CTC residues from muscle, liver and kidney of broilers.
Table 1. Maximum Residue Limit of CTC hydrochloride in the target tissues of food producing animals.

<table>
<thead>
<tr>
<th>Pharmacologically active substance(s)</th>
<th>Marker residue</th>
<th>Animal species</th>
<th>MRLs</th>
<th>Target tissues</th>
<th>Other provisions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>Sum of parent drug and it’s 4-epimer</td>
<td>All food producing species</td>
<td>600 μg/kg</td>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td></td>
<td></td>
<td>300 μg/kg</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td></td>
<td></td>
<td>100 μg/kg</td>
<td>Muscle</td>
<td>-0-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 μg/kg</td>
<td>Milk</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200 μg/kg</td>
<td>Eggs</td>
<td></td>
</tr>
</tbody>
</table>

Commission Regulation (EC) 508/1999

Table 2. Body weight of the broilers during the whole experiment

<table>
<thead>
<tr>
<th>Feed with CTC</th>
<th>Days of the experiment</th>
<th>Broilers’ carcass weight of the biological experiment (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day</td>
<td>1260 1335 1570 1180 1390 1380</td>
<td></td>
</tr>
<tr>
<td>3rd day</td>
<td>1550 1440 1340 1700 1300 1300</td>
<td></td>
</tr>
<tr>
<td>5th day</td>
<td>1700 2000 1750 1700 1350 1700</td>
<td></td>
</tr>
<tr>
<td>Feed without CTC</td>
<td>1st day</td>
<td>1900 1400 1780 1750 1700 1500</td>
</tr>
<tr>
<td>2nd day</td>
<td>1800 1600 1800 1580 1650 2000</td>
<td></td>
</tr>
<tr>
<td>3rd day</td>
<td>1600 1650 1850 1900 1900 1750</td>
<td></td>
</tr>
<tr>
<td>5th day</td>
<td>2000 1750 1550 1900 2000 2000</td>
<td></td>
</tr>
<tr>
<td>10th day</td>
<td>1900 2100 1800 2300 2400 2100</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2300 1900 1600 1900 1500 1700</td>
<td></td>
</tr>
</tbody>
</table>