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# Chloramphenicol Residues in Muscle of Rainbow Trout Following Two Different Dose Treatments

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**Abstract** Depletion of chloramphenicol (CAP) in muscle of rainbow trout was evaluated following 4 days of oral administration with two dosages (42 and 84 mg/kg/day). Sampling was conducted during treatment and for 35 days following the end of treatment. Analysis was carried out using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Concentrations observed during treatment were more than 300 μg/kg. A significant elimination occurred within 9 days after the cessation of treatment in both groups. Higher CAP levels were measured in the group treated with higher dose. CAP was not detectable after 13 and 15 after the end of treatment in both groups.

**Keywords** Chloramphenicol · Trout · Elimination · Liquid chromatography–tandem mass spectrometry

Chloramphenicol (CAP) is a broad-spectrum antibiotic with bacteriostatic properties effective against a wide range of Gram-positive and Gram-negative bacteria. CAP is absorbed in the gastrointestinal tract and its half-time elimination varies widely among species, and is primarily due to hepatic metabolism, while its metabolites are inactive and excreted in the urine. Its lipid solubility enables the molecule to persist in most tissues, body fluids and the central nervous system, and it readily diffuses to milk and eggs (Dowling 2006). It has been suggested that differences in pharmacokinetic parameters among species may be due to different anatomical volumes, plasma proteins and tissue binding of a drug (Weifen et al. 2004). From the toxicological perspective, CAP is a cytotoxic, genotoxic and haemotoxic compound that can cause bone-marrow depression, a disorder that is reversible after drug withdrawal, as well as serious and irreversible aplastic anemia that can result in leukaemia (Holt and Bajoria 1999).

Despite regulations prohibiting the use of CAP in food-producing animals in the European Union since 1994, CAP residues can be found in samples taken as part of national monitoring plans in all European countries and third countries, especially in shrimp and other aquatic animals like fishes (WHO 2004). After oral intake of CAP at a certain dose, the drug is completely absorbed and well distributed to all tissues, achieving the therapeutic dose. CAP levels in blood are proportional to the applied doses. Fish can be treated with CAP orally as medicated feed in a concentration of 4–8 g per 100 kg. Therefore, the Food Standards Agency of Ireland (2002/2003) in 2002 presented CAP concentrations in the range from 0.1 to 34 μg/kg in samples of aquaculture products (WHO 2004). In the previous evaluation by the Committee in shrimp samples, the presence of CAP was determined as mean value of 0.25 μg/kg (range, 0.06–0.69 μg/kg), with two outlying values of 3.0 and 3.7 μg/kg. It is suspected that there are two sources of residues in food; very low concentrations can be the result of environmental contamination, while higher concentrations can be explained by intentional use

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of the drug and false monitoring of withdrawal times, which can be very different among animal species.

Rainbow trout is the mostly cultivated freshwater species in aquaculture as it inhabits many freshwater lakes, is adapted for intensive farming and prized in recreational fishing. Fish production increases annually, which can be connected with the increasing awareness of the nutrition benefits from consuming such food. Inland production of rainbow trout to supply domestic markets has increased in countries such as Italy, France, Germany, Denmark and Spain. The largest producing countries include the USA, Iran, Germany and the United Kingdom (FAO 2009). In the EU, the concept of the minimum required performance limit (MRPL) has been established for screening and the confirmatory method used for for substances for which no permitted limit has been established (EC 2003). Minimum required performance limit (MRPL) means minimum content of an analyte in a sample, which at least has to be detected and confirmed. The Commission Decision set the MRPL for CAP at 0.3 μg/kg in foods of animal origin, including aquaculture products. The literature contains few publications regarding CAP depletion in fish species. According to different experimental condition such as usage of different aquatic species and species sizes, tank water temperatures, CAP doses used and duration of treatments, it is difficult to deduce main factor affecting the CAP depletion (Weifen et al. 2004; Huang et al. 2006; Biancotto et al. 2009). Various analytical conditions have been applied for the determination of CAP by liquid chromatography–tandem mass spectrometry in animal tissues, shrimp tissue and rainbow trout (Impens et al. 2003; Ashwin et al. 2005; Santos et al. 2005; Biancotto et al. 2009).

The aim of this study is to determine the withdrawal time of CAP in rainbow trout after treatments with feed medicated with two different doses. CAP concentrations were determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using validated in-house method.

## Materials and Methods

Rainbow trout (*Oncorhynchus mykiss*) was cultivated in two separate pools, each containing 50 kg of biomass (200 fish, mean weight 250 g). Pools were fibreglass; outdoor tanks with a volume of 4.7 $m^3$, and fish were kept under natural daylight conditions. Tanks were supplied with a constant flow of water (720 L/h) at a temperature of 9.9 ± 0.1°C, pH 7.5 ± 0.1 and an oxygen content of 92.8 ± 3.1 %.

The CAP was administered over 4 days with a dose of 42 mg/kg/day in the first pool and double dose of 84 mg/kg/day in the second. Feed was incurred with a drug solution (150 mg/mL; Jiangxi Dongxu Chemical Technology Ltd., China) and well mixed to 500 g of pellet feed to achieve homogeneity of the drug through the matrix. For a daily therapeutic dose of 42 mg/kg/day over 4 days, 14 mL of solution was added to the daily amount of feed for the first group. For the second test group, 28 mL of the CAP solution was added to 500 g of feed, i.e. the dose was 84 mg/kg/day. Fish were fed twice daily with 0.5 kg of medicated pellets per 100 kg of fish.

Sampling was carried out parallel in both tanks on the day preceding the start of treatment (day −1), on days 2 and 4 of treatment, and on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 35 after the end of treatment (i.e. days 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 etc. of the study).

On each sampling day, 3 fish were taken from each pool, with special care to avoid cross contamination. In sampling process skin and internal organs were removed. Muscle tissues and subcutaneous fat were sampled and partially homogenized. Samples were packed in plastic bags and stored at −18°C until analysis.

Water from the pools was also sampled and stored in the same manner, but was found not to contain traces of CAP.

Dichloromethane, petrol ether and ammonium acetate, as ultra resin analysis chemicals, methanol and diethyl ether of LC–MS grade and octadecyl ($C_{18}$) solid phase extraction (SPE) cartridges (500 mg per 3 mL) were purchased from J. T. Baker (Deventer, Netherlands). Acetone (for LC) and monopotassium phosphate (p.a.) were supplied by Merck (Darmstadt, Germany), sodium sulphate (anhydrous) by Carlo Erba (Milan, Italy) and sodium chloride and disodium hydrogen phosphate dihydrate by Kemika (Zagreb, Croatia). Nitrogen 5.0 and 5.5 were purchased from SOL spa (Monza, Italy). Ultra pure water was obtained Milli-Q system (Millipore, Bedford, USA). The 5 mM ammonium acetate ($CH_3COONH_4$) solution was prepared by diluting 0.38 g $NH_4OAC$ in 1 l ultra pure water and was used as mobile phase A. Mobile phase B was methanol of LC–MS grade.

The CAP standard used was from Sigma-Aldrich (St. Louis, MO, USA) and the internal standard d5-CAP was purchased from the Cambridge Isotope Laboratory (Andover, MA, USA). CAP standard stock solutions were prepared in methanol at concentrations of 10 μg/mL, 100 ng/mL and 10 ng/mL. The d5-CAP stock solution was prepared at a concentration of 100 ng/mL, and used for matrix fortification. Further dilutions at concentrations of 0.625, 1.25, 2.5, 5 and 10 ng/mL in mobile phase A and B (1:1) were made for calibration curves. Each calibration standard included the internal standard at 2 ng/mL.

The following equipment was used in sample preparation: Waring Commercial Blender 7011HS (Waring Commercial, Connecticut, USA), Kinematica dispersing

**Table 1** MS/MS conditions for MRM analysis of CAP and d5-CAP (italicized transitions are quantifier product ions)

| Analyte | Precursor ion | Product ion | Fragmentor (V) | Collision energy (V) |
|---|---|---|---|---|
| CAP | 321 | *152* | 110 | 13 |
| | 321 | 257 | 110 | 5 |
| d5-CAP | 326 | *157* | 100 | 13 |
| | 326 | 262 | 100 | 9 |

system, Polytron model T-2000 (Kinematica, Inc., Switzerland) and IKA® Vortex model MS2 Minishaker (IKA® WERKE GMBH & CO.KG, Staufen, Germany), Iskra ultrasonic bath (ISKRA PIO, d.o.o., Slovenia), Supelco vacuum manifold (Supelco Inc, Bellefonte, PA, USA), centrifuge Rotanta 460R (Hettich zentrifugen, Tuttlingen, Germany) and Nitrogen evaporation system N-EVAP™ model 112 (Orgamonation Associates Inc., USA).

The LC–MS/MS system consisted of HPLC Agilent Technology 1200 and Triple Quad LC/MS 6410 mass spectrometer (Agilent, USA). Chromatographic separation was achieved by isocratic elution on the Zorbax XDB C18 column 4.6 × 75 mm, 3.5 μm (Agilent, USA) with 30 % 5 mM ammonium acetate and 70 % methanol.

The triplequad mass spectrometer consisted of an ESI ion source and was operated in negative mode. The following MS–MS parameters were used: desolvation temperature, 350°C; nebulizer and dryer gas at 5 L/min and 35 psi; capillary voltage, 4,000 V; chamber current 0.25 μA; high purity nitrogen as collision gas, cell exit voltage 8 V; Cell RF voltage 400 V. Dwell time for all single MRMs were 80. The injection volume was 15 μL and mobile phase flow was 0.5 mL/min. One chromatographic run was 5 min, with an additional 2 min for column stabilization. Column temperature was 40°C. The MS/MS conditions for Multiple Reaction Monitoring (MRM) analysis by selecting the most intense ion transition from the CAP and d5-CAP is reported in Table 1.

A total of 10 g of the homogenized fish sample was weighed and fortificated at 0.4 μg/kg d5-CAP, which corresponds to 2 μg/L d5-CAP in the final extract. The internal standard was added to samples containing CAP at doses of the calibration range after extract dilution. Extraction of CAP from the matrix was achieved by adding 50 mL of the aceton/dichlormethan mixture (1:1, v/v) and mixing with the dispersing system for 1 min. To remove water from the mixture, 10 g sodium chloride and 10 g sodium sulphate were added. After each addition, samples were shaken for 30 s.

After centrifugation (3,000*g*, 5 min), 5 mL of supernatant was taken and evaporated with nitrogen until dry at 50°C. CAP in pH 7.8, with 2.5 mL phosphate buffer adjusted, is deprotonated and charged negatively. $C_{18}$ SPE columns were previously activated with 2.5 mL methanol and 5 mL water. Samples were added to the column and deprotonated CAP molecules were trapped by the activated $C_{18}$ groups inside the column. Columns were washed by adding 5 mL water in the sample tube, and 5 mL directly to the column. Columns were dried at high vacuum for 15 min to remove water residues. Any non-polar impurities were washed by adding 5 mL petrol ether. After a short drying process, CAP was eluted with 10 mL diethyl ether. Samples were further evaporated with nitrogen at 50°C. Residues were dissolved with 100 μL methanol and 100 μL of 5 mM ammonium acetate and vortexed. Samples were filtered through 0.45 μm regenerated cellulose membrane filters prior to injection in the LC–MS/MS.

Because of the high concentrations of CAP, samples are needed to be additionally diluted (1:100) to fit the calibration range. In that case internal standard was added to the sample in the final dilution step to achieve a concentration of 2 μg/kg, same as in calibration standards.

The method was carried out according to the criteria laid down by the Commission Decision 2002/657/EC (EC 2002). Specificity was tested by analyzing 20 representative blank fish muscle samples in order to verify the absence of any interfering peaks at the CAP retention time. Limit of detection LOD was obtained by adding 3 and 10 times the standard deviation of the 20 blank samples to the mean blank value. Response linearity was calculated from the six point calibration curve (0.625; 1.25; 2.5; 5; 10 μg/kg and fixed 2 μg d5-CAP/l).

Method trueness and precision was determined by fortifying blank muscle samples with CAP at 0.15, 0.3, and 0.45 μg/kg in six replicates for each level and analyzed on three different days. The mean recovery and RSD was calculated for each fortification level (0.15; 0.3 and 0.45 μg/kg). Decision limit and detection capability were calculated by applying the calibration curve procedure. The decision limit (CC$\alpha$) was expressed as the concentration corresponding to the lowest calibration level (0.15 μg/kg) plus 2.33-fold the within-laboratory standard deviation calculated at this level ($n = 18$). The detection capability (CC$\beta$) was calculated as CC$\alpha$ plus 1.64-fold the standard deviation of the within-laboratory reproducibility at all levels ($n = 54$).

Statistical analyses were performed using the Statistica® 6.1 software package (StatSoft® Inc., USA). Results were expressed as mean ± SD. Observations were analyzed by the analysis of variance. Differences in chloramphenicol concentration between two treated groups on the same day of determination were analyzed by Student's $t$ test for independent pairs. Statistical differences taken at $p < 0.05$ were considered significant.

**Table 2** Trueness (recovery) and precision of the method

| Fortification level (μg/kg) | Measured concentration (μg/kg) | Recovery ± SD (%) | RSD (%) |
|---|---|---|---|
| 0.15 | 0.153 | 102.2 ± 10.7 | 10.5 |
| 0.3 | 0.290 | 96.6 ± 7.3 | 7.6 |
| 0.45 | 0.456 | 101.3 ± 6.4 | 6.3 |

## Results and Discussion

In this study, rainbow trout was treated with CAP at two dose levels and the withdrawal period was estimated over 35 days after the end of treatment.

During every batch of analyses, quality control samples (a blank trout muscle and a blank trout muscle fortified at 0.3 μg/kg) were processed to verify the absence of in-lab sample contamination and to control recovery rates. All control sample recoveries were within the interval of −70 % to +10 %. CAP quantization was accomplished using the isotope dilution method considering the most intense ion transition. Quality control in the quantitation process included requirements for the response factor (RSD% < 23 %), ion ratio (tolerance 20 %) and relative retention time (tolerance 2.5 % of the RRT). Also, the internal standard recovery used for matrix compensation was higher than the set limit (25 %).

In the validation study, the following parameters were determined: limit of detection LOD = 0.07 μg/kg, decision limit CCα = 0.171 μg/kg and detection capability CCß = 0.188 μg/kg. Table 2 gives details of trueness (recovery, Rec %) and precision (standard deviation, RSD %) of the method. Validation parameters indicated that the method is appropriate for the detection of CAP at low levels. Figure 1 shows the chromatogram and mass spectrum corresponding to trout muscle sampled on the first day after the end of treatment (i.e. on day 5, with internal standard added).

Table 3 provides the given estimated concentration of CAP in both experimental groups of trout prior to, during and after the end of treatment. Data showed higher CAP levels during treatment and within the first day of elimination in group treated with dose 84 mg/kg/day, i.e. fish subjected to treatment with higher CAP dose. However, there were no significant differences between the two experimental groups.

On the second day of the treatment (day 2) CAP was distributed to muscle reaching maximum doses of 708.2 μg/kg in group treated with dose 42 mg/kg/day and 1031.3 μg/kg in group treated with dose 84 mg/kg/day. However, on day 4 of treatment, the measured levels were

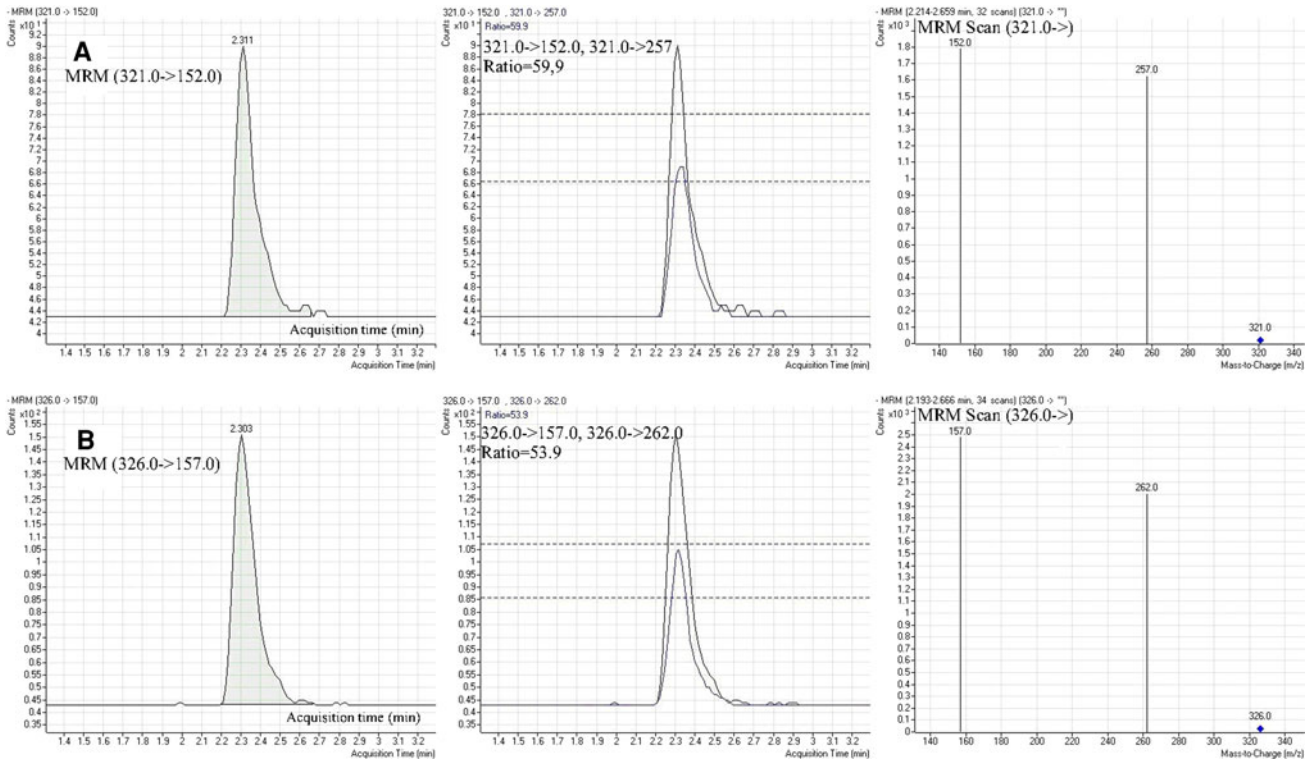


**Fig. 1** Chromatograms and mass spectrum results (**A** for chloramphenicol and **B** for internal standard) of the trout muscle sampled on the first day after the end of treatment spiked with internal standard at 2 μg/kg

**Table 3** Concentrations of chloramphenicol (range, mean ± SD, n = 3) in trout muscle before, during and after treatment by medicated feed with two doses (A = 42 mg/kg/day; B = 84 mg/kg/day)

| Days | Dose: 42 mg/kg/day | | Dose: 84 mg/kg/day | |
|---|---|---|---|---|
| | Range (μg/kg) | Mean ± SD (μg/kg) | Range (μg/kg) | Mean ± SD (μg/kg) |
| Before treatment | | | | |
| −1 | | ND | | ND |
| Treatment | | | | |
| 1 | | NM | | NM |
| 2 | 422.3–708.2 | 612.9 ± 202.1 | 1031.3–834.7 | 872.8 ± 120.4 |
| 3 | | NM | | NM |
| 4 | 193.8–365.9 | 308.5 ± 121.7 | 364.1–707.7 | 593.1 ± 242.9 |
| Withdrawal time | | | | |
| 5 | 213.8–332.5 | 292.9 ± 83.9 | 273.9–523.1 | 440.1 ± 176.2 |
| 7 | 23.7–32.7 | 28.2 ± 6.39 | 22.9–32.4 | 26.8 ± 5.02 |
| 9 | 0.64–1.54 | 1.09 ± 0.45 | 0.90–1.10 | 1.03 ± 0.11 |
| 11 | 0.28–0.42 | 0.37 ± 0.10 | 0.25–0.46 | 0.31 ± 0.11 |
| 13 | 0.068–0.18 | 0.11 ± 0.06 | 0.05–0.18 | 0.13 ± 0.09 |
| 15 | 0.044–0.059 | 0.05 ± 0.01 | 0.074–0.09 | 0.08 ± 0.01 |
| 17 | 0.031–0.04 | 0.04 ± 0.01 | 0.05–0.061 | 0.06 ± 0.01 |
| 19 | | ND | 0.02–0.034 | 0.03 ± 0.01 |
| 21 | | ND | | ND |
| 23 | | ND | | ND |

*NM* not measured, *ND* not detected

1.5–2 times lower than those measured on day 2 in both experimental groups.

The first day after treatment, concentrations of CAP were more than 250 μg/kg in both groups of animals. Then, a significant elimination of CAP occurred within 9 days after the cessation of treatment in both treated groups. Also, on day 9 after the end of treatment, measured CAP concentrations were below 0.3 μg/kg (MRPL value). CAP levels were not detectable on day 13 after the end of treatment in fish samples of group treated with dose 42 mg/kg/day and on day 15 in group treated with dose 84 mg/kg/day.

In present study, related to the variation of feed intake among fishes wide variation in drug concentrations were determined and the exact elimination half-time cannot be calculated. The effects of social status and socially induced differences in food consumption between dominant and subordinate fish may be responsible at least in part for the lower growth rates (DiBattista et al. 2006). In previous investigations lower growth rates in subordinate fish suggests that a metabolic disadvantage is associated with s monopolization of food sources by dominant fish individuals, as well as appetite suppression and changes in overall metabolic capacity (Sloman et al. 2000). The results obtained in this study were much lower than CAP levels measured in a previous study on rainbow trout (Biancotto et al. 2009). In that study, a CAP dosage of 73.9 mg/kg/day was administered to trout over 10 days kept in tanks with constant flow of 8 L/h, as opposed to the dosages of 42 and 84 mg/kg/day over 4 days in tanks with much higher water flow (720 L/h) used in this study. Accordingly, in the present study, elimination occurs rapidly over 10 days, while in the previous study CAP levels were measured at 0.3 μg/kg level even after 31 days (Biancotto et al. 2009). A similar study found that the elimination of CAP in shrimp (*Penaeus chinensis*) lasted 3 days (Weifen et al. 2004), and thus it can be affirmed that withdrawal periods vary extremely among species (Pengov et al. 2005).

In conclusion, results presented are dificult to compare to those from the literature consulted on different fish species, different drug concentration in diet and intake of doses or environmental factors such as temperature.

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