

Residue Withdrawal of Florfenicol from the Serum and Edible Tissues of Broiler Chickens

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Abstract: Florfenicol (FFC) is a synthetic antibiotic with a broad antibacterial spectrum and therapeutic effectiveness. It was specifically developed for veterinary use, with proven value in the treatment of respiratory and enteric infections in food animals. It is active against many chloramphenicol and thiamphenicol-resistant strains. In the present study, serum and tissue residual level of FFC and histopathological investigation of liver tissue after oral administration of 30 mg/kg bw and 60 mg/kg bw (5 consecutive days) to broiler chickens were studied. A validated high performance liquid chromatography (HPLC) method with photodiode array detector (PAD) at a wavelength of 223 nm was used for the determination of FFC concentration. Samples were extracted with ethyl acetate and defatted with hexane, followed by clean-up and chromatographic separation on hypersil gold (C18) column with acetonitrile: water as a mobile phase. The residual analysis resulted that FFC concentration in liver was higher than the concurrent serum and muscle concentrations. On 7th day, although the level of FFC was very low, but still detectable in the serum and muscle, on the other hand the concentration in liver declined but still high especially in 60 mg/kg bw treated group. FFC tissue and serum level in 60 mg/kg bw treated group was significantly increased compared with that of the 30 mg/kg bw treated group. Liver FFC level was below the maximum residue limits (MRLs) in both treated groups all over the studied time course, while muscle tissue FFC level was higher than the MRLs at 3rd day post administration, then the level depleted to be below the MRLs at 5th day in the 30 mg/kg bw treated group and at 7th day in the 60 mg/kg bw treated group. The histopathological investigation showed microscopically alterations in liver tissue which appeared to be more serious in 60 mg/kg bw treated groups than that received the therapeutic dose (30 mg/kg bw). The current study concluded that it is necessary to ensure that the residues of FFC were less than the MRLs to guarantee its safety to consumer.

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1. Introduction

In various types of food producing animals, the abuse and overuse of veterinary drugs especially antibiotics resulted in the presence of these compounds in final products for human consumption, including meat, fish, milk, poultry, liver, eggs and even honey (Becker *et al.*, 2004; Berrada *et al.*, 2008; Carretero *et al.*, 2008; Hermo *et al.*, 2008 and Bogillai *et al.*, 2009). Therefore, monitoring of antibiotic residues in feed is of importance for controlling contamination within the food processing chain, potentially resulting in deleterious health effects in animals and consumers, and violating good manufacturing guidelines.

Because of health hazards related to drug residues in animal tissues which increased risk of developing of antibiotic-resistant bacteria and allergies in individuals with hyper-sensitivity

(Hardy, 2002 and Woodward, 2005), many countries including China, USA, Japan and member states of European Union Commission (EUC) strictly reserved them for the prevention or treatment of diseases (Epstein *et al.*, 1994 and EC, 2003). However, antibiotics might still make their way into animal based food products, either by illegal usage through antibiotic containing feeding stuffs, or unintentional cross contamination.

Florfenicol (FFC), a fluor-derivative of chloramphenicol and thiamphenicol, was developed in the United States by Schering-Plough Animal Health (now Merck & Co Inc.). FFC is a more secure drug compared to chloramphenicol. Hence, it overcomes the potential fatal side effects of chloramphenicol (human aplastic anemia, bone marrow suppression and Grey Baby Syndrome) through the substitution of nitro group attached to the

benzene ring by a sulfomethyl group (Johnson, 2003).

The mechanism of antibacterial activity of FFC is the same as that of thiamphenicol and chloramphenicol. It inhibits bacterial protein synthesis at the ribosome by binding at the A site of the peptidyl transferase center where it perturbs the placement of A site tRNA and thus prevents the peptide bond formation and is generally considered to be bacteriostatic antibiotics (Cannon *et al.*, 1990).

The structural modification of FFC including substitution of a fluorine atom instead of the hydroxyl group located at C-3 in FFC (Sams, 1994) prevents a bacterial enzymatic acetylation at this site which induced by acetyltransferase, an enzyme used by bacteria to develop resistance (Cannon *et al.*, 1990; Paape *et al.*, 1990). Consequently, FFC has more antibacterial activity compared with chloramphenicol and thiamphenicol, including activity against many isolates resistant to chloramphenicol such as *Escherichia coli* (Cannon *et al.*, 1990), *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella typhimurium* (Afifi and Abo el-Sooud, 1997) *Staphylococcus aureus*, *Pasteurella spp.* (Marshall *et al.*, 1996), *Actinobacillus pleuropneumoniae* (Ueda *et al.*, 1995) and *Mycoplasma mycoides* (Ayling *et al.*, 2000).

Formerly, it was mainly used in aquaculture, swine and cattle bacterial disease treatments such as respiratory diseases and/or foot rot. However and more recently, it was emerged to the poultry industry to enhance the health and the productivity of the flocks (Park *et al.*, 2008 and Koc *et al.*, 2009). A very few reports discussed the dosage, pharmacokinetics and the residual effect of FFC in chicken (Shen *et al.*, 2002, El-Banna and El-Zorba 2011). However, proper toxicological and residue studies should be conducted before its overuse in the clinical application of broiler chickens.

At the beginning of the 21st century, high-performance liquid chromatography (HPLC) was used frequently for drug determination in biological fluids and tissues in many clinical laboratories, considered as an established technique with highly automated instrumentation and with concentration sensitivity in the nanomolar range, as it is ideal for bioanalytical assays (Kowalski *et al.*, 2005).

The objective of this study was to provide tissue residue data necessary to fulfill the human food safety requirements of FFC use in broilers. This was done by evaluation of FFC withdrawal

time in serum and tissues (muscle and liver) of broiler chickens after its oral administrations for 5 consecutive days, besides the histopathological investigation of liver tissue.

2. Materials and Methods

Animal

Thirty (one day old) Hubbard chicks were used in this study. The birds were supplied from EL-Dakhliya Company and were placed in the Experimental Animal House of Faculty of Veterinary Medicine, Zagazig University. The chickens were monitored for any apparent clinical signs of disease before drug administration. The chicks had free access to water and antibacterial-free food *ad libitum* daily. Vaccination programs against Newcastle and Gumboro diseases were applied.

Drug and Chemicals

Acetonitrile (Lab-scan), Ethyl acetate (Lab-scan), Hexane (Lichrosolv), Sodium chloride and Sodium sulfate were all HPLC grade and were used as received without any further purification. Phosphoric buffer solution (pH = 6.88) was prepared from the mixture of 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 solutions. Florfenicol standard was purchased from Sigma-Aldrich Co. (St. Louis, Mo) and stock solution of 30mg/mL florfenicol was prepared by dissolving substance in polyethylene glycol at 100 mg/mL for oral dosing.

Analytical method

Florfenicol residues in serum, muscle and liver tissues were analyzed with reverse phase (HPLC) system, containing a photodiode array detector (PAD) (Thermo Scientific Co., USA). The separation was accomplished on hypersil gold C18 (10 μm , 100x4.6 mm) column at 30°C. The mobile phase was acetonitrile -Water (HPLC grade) at a ratio 27:73 (v/v), no buffers or mobile phase modifiers were added. Aliquots of Twenty microliters were injected on the HPLC column and monitored at a wavelength of 223 nm, the flow rate was 1.0 mL/min.

Experimental design

Thirty broiler chickens (20 days old) were randomly divided into three groups of 10 birds. Chickens of group 1 were given 5 oral doses of FFC over 5 successive days at a dose level of 30 mg/kg bw as a therapeutic dose (Shen *et al.*, 2003). Whereas chickens of group 2 were given the drug at a dose level of 60 mg/kg bw as a double therapeutic dose at the same manner. The remaining 10 chickens (group 3) did not receive the drug and were kept as a control group. Chickens were weighed prior to drug

administration and the doses were calculated accordingly. FFC was administered directly into the crop using a thin plastic tube attached to a syringe. Food was withheld for 12h before and 6h after drug administration and water was provided *ad libitum*.

Samples collection

After the course of treatment, the chickens were bled at 3rd, 5th and 7th days post last dosage. All blood samples were centrifuged directly at 1000 rpm for 5 min and serum was harvested and stored at -20°C for further analysis. At necropsy of birds, muscle and liver tissues were sampled at the same order of serum collection and partially homogenized, packed in plastic bags and stored at -20°C until further analyzed.

Sample preparation

1. Extraction

FFC was extracted from tissue and analyzed as described by **Shen and Jiang (2005) and Lewbart *et al.* (2005)**. Ten grams of ground chicken muscle and liver tissues were weighed into a plastic centrifuge tubes. 10 mL of Phosphoric buffer solution (pH = 6.88), 3 gm sodium sulfate, 10 mL ethyl acetate were added to the centrifuge tube. The mixture was shaken vigorously for 10 min. After centrifugation for 5 min at 5500 rpm, the supernatant was transferred into another tube. The previous extraction step was repeated once. The extracts were evaporated to dryness under nitrogen stream in a water bath at 45–50°C. 2 milliliters of methanol was added, and the tube then vortexed for about 30 seconds. Thereafter, ten mL of 4% NaCl and 20 mL of hexane were added into the tube. This mixture was then vigorously shaken for about 30 seconds. Then allowed to separate for several, the hexane layer was discarded by aspiration. The de-fatting step was repeated and the extract was again evaporated to dryness with a gentle stream of nitrogen in a water bath at 45–50 °C.

The serum samples (1 mL) were extracted twice with ethyl acetate and evaporated to dryness as previously mentioned.

2. Clean-up

Before analysis, the obtained residues were reconstituted in 2 mL of mobile phase, vortexed and poured through a 0.45 µm nylon centrifuge filter into an auto-sampler vial. The extract was then ready for analysis.

Calibration curve

The calibration curve was prepared on the basis of the peak areas and the working solution concentrations. A series of working standard florfenicol solutions at concentrations of 10.0,

5.0, 2.5, 1.0, 0.5, 0.25, 0.10, 0.05, 0.01 µg/mL which were prepared by diluting the stock solutions with acetonitrile–water (25:75), then they injected into HPLC and analyzed.

Method validation

Selectivity and Sensitivity:

No interference was observed at the retention time of the analyzed samples. The retention time of FFC was 3.4 minutes.

Accuracy and Precision:

The recovery was determined by repetitively analyzing blank chicken liver, muscle and serum spiked with known concentration of FFC. The accuracy percent recovery of the method ranged from 84%-90% for the liver, 77-81% for muscle and 89-97% for the serum samples with relative standard deviations indicating precision (% RSD) of 0.21%, 0.54% and 0.37%.

Linearity:

The calibration curve was calculated by linear regression equation method as $Y = 4E - 06X - 0.03$ ($r^2 = 0.999$). The X symbol indicated area under peak and Y indicated the concentration of FFC. The correlation coefficient was 0.999, which showed a good linearity within the range of 0.039 to 2.5 µg/mL.

Histopathological Studies

Specimens from the liver were collected after necropsy at 3rd, 5th and 7th days post last administration and fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared, stained by H&E and examined microscopically for histopathological examination (**Bancroft and Gamble, 2008**).

Statistical analysis

The results were displayed as mean±SE and Student (*t*) test (**Snedicor and Cochran, 1987**).

3. Results and Discussion

Although FFC is currently approved for use in veterinary medicine, its usage in chicken species is still limited due to the concern of conferred antimicrobial resistance by preexisting genes (floR, cfc or fexA) in avian species (**Kehrenberg & Schwarz, 2006**). However, the superiority of FFC over other amphenicols and the availability of the drug as a feed additive have prompted the need for more residual data to safeguard against improper use of the drug.

HPLC is a well-established technique for measuring solutes in the biological fluids and tissues of laboratory animals and mammals, it is ideal for bioanalytical assays (**Kowalski *et al.*, 2005**).

In this study, broiler chickens were treated with FFC at two dose levels of 30 mg/kg bw and 60 mg/kg bw as a therapeutic and double therapeutic dose respectively. Hence, it was suggested by Shen and his colleagues that FFC should be given at a dosage of 30 mg/kg bw to maintain therapeutic concentrations (Shen *et al.*, 2003) and the withdrawal time was estimated

over 7 days after the end of treatment. Residues withdrawal of FFC in various tissues of broiler chickens (serum and tissues) in both treated groups following drug administration at different times are represented in Figure (1) and Table (1), the incurred tissue chromatograms are presented in Figure (2).

Table 1: Florfenicol Serum and Tissues (muscle and liver) Concentration in Broiler Chickens Following Oral Administration at 30 mg/kg bw and 60 mg/kg bw for 5 Successive Days (mean \pm S.E, n=3)

Serum & Tissues	Days post last dosage	Dose	
		30 mg/kg bw	60 mg/kg bw
Serum ($\mu\text{g/ml}$)	3	0.059 \pm 0.01	0.35 \pm 0.06
	5	0.045 \pm 0.00	0.13 \pm 0.02*
	7	0.04 \pm 0.01	0.06 \pm 0.01*
Muscle ($\mu\text{g/gm}$)	3	0.14 \pm 0.01	0.32 \pm 0.05
	5	0.09 \pm 0.00*	0.14 \pm 0.02*
	7	0.052 \pm 0.01*	0.10 \pm 0.00*
Liver ($\mu\text{g/gm}$)	3	0.89 \pm 0.07	1.66 \pm 0.19
	5	0.48 \pm 0.05*	0.85 \pm 0.07*
	7	0.11 \pm 0.02**	0.66 \pm 0.04**

*Significant at $p \leq 0.05$

On the 3rd day after the last dosage, FFC was distributed to muscle and liver reaching to (0.14 \pm 0.01 and 0.89 \pm 0.07) respectively in 30 mg/kg bw treated group and (0.32 \pm 0.05 and 1.66 \pm 0.19) in 60 mg/kg bw. From the previous findings, it is clear that the highest level was observed in liver tissue in both groups. On the 5th day post last dosage, the measured level was significantly decreased in muscle and liver tissues in compared with those measured on the 3rd day in both experimental groups (nearly 1.5-2 times). On the 7th day, although the level of FFC was very low, but it was still detectable in the serum and muscle (0.04 \pm 0.01 and 0.052 \pm 0.01 respectively in 30 mg/kg bw treated group) and (0.06 \pm 0.01 and 0.10 \pm 0.00 in 60 mg/kg bw treated group). On the other hand, the concentration in liver tissue was significantly declined but it was still high especially in the 60 mg/kg bw treated group. In this study, it is obvious that the concentration of FFC in the tissues were higher when compared with the concurrent serum concentration. This can be attributed to the relatively low affinity of serum protein binding to FFC which leads to the extensive disposition of the drug in highly

prefused organs/ tissues such as liver (Chang *et al.*, 2009). Other factors can explain the plasma-to-tissue gradient of FFC were that for certain drugs, unrestricted diffusion across capillaries cannot be taken for granted. Physiological factors that affect blood-to-interstitium transfer, such as local blood flow, local capillary density (Snyder, 1995), and capillary permeability, may all have influences. However, our finding of the considerable high FFC level in liver tissue was also previously reported in broiler chickens (Afifi and Abo El-Sooud, 1997; El-Banna *et al.*, 2007 and El-Banna and El-Zorba, 2011) and in ducks (El-Banna, 1998).

Up till 7 days post drug administration, the FFC concentration in serum, muscle and liver tissues declined but it was still detectable. This was clear in the both treated groups especially in the 60 mg/kg bw treated group. These results were supported by a previous report (Anado'n *et al.*, 2008) where they found that following multiple oral doses of 40 mg/ kg for 3 days, tissue disposition of FFC were persisted up to 7 days. However, in previous study, the concentration of FFC was not detectable in any of the chicken tissues 72 h after drug

administration (Chang *et al.*, 2009). The difference between the withdrawal curves of FFC in the different studies can be explained by the differences of FFC pharmacokinetics in the

different chicken breeds. Hence, potential breed differences in FFC disposition were recorded before between Leghorn chickens and Taiwan Native chickens (Chang *et al.*, 2009).

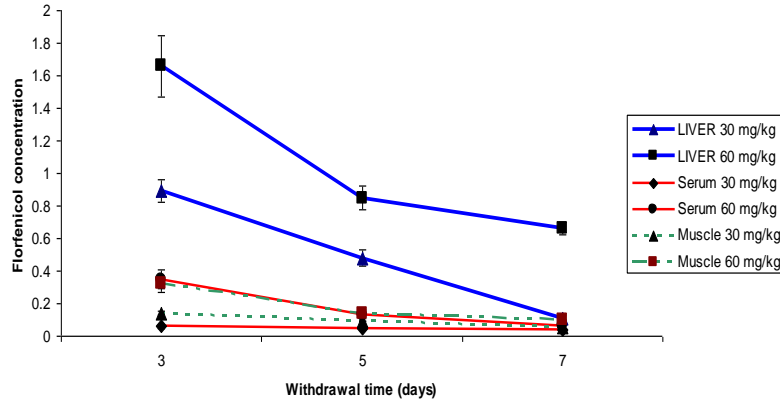


Figure 1: Residue Withdrawal Curve for Florfenicol in Serum and Tissues (muscle and liver) of Broiler Chickens Following Oral Administration of 30 mg/kg bw and 60 mg/kg bw for 5 Successive Days (mean \pm S.E, n=3)

Regarding to the FFC level in both treated groups, our results revealed that the FFC tissue and serum level in 60 mg/kg bw treated group was significantly increased compared with that of the 30 mg/kg bw treated group (Figure1), this indicated that FFC is removed from the body at a slower rate when dosed at the higher level (Shen *et al.*, 2003). Chang *et al.* (2009) concluded that the higher doses or multiple doses may result in drug residues being detected for a longer period of time and the withdrawal time should therefore be extended.

According to the Committee for Veterinary Medicine Products (CVMP) in the European Agency for the Evaluation of Medicinal Products (EMA, 2002), the maximum residue limits (MRLs) already set in relevant species (e.g. chicken) can be extrapolated to the edible tissues (muscle and liver) were 100 $\mu\text{g}/\text{kg}$ and 2500 $\mu\text{g}/\text{kg}$ respectively. In this study, mean liver FFC level was not above the MRLs in both treated groups all over the post dosing period, while muscle tissue FFC level was higher than the MRLs at 3rd day post administration, thereafter the level depleted to be below the MRLs at 5th day in the 30 mg/kg bw treated group and at 7th day in the

60 mg/kg bw treated group (Figure 3). This residual withdrawal data suggesting that FFC is considered safe for human consumption after 5 days withdrawal time when used at a level of 30 mg/kg bw and after 7 days withdrawal time when used at a level of 60 mg/kg bw.

Pharmacochemicals usage in animal farming disease treatment can cause morphological and functional changes in absorption capacity, metabolism and excretion organs, causing alterations on the organs. The liver is a critical organ which contains most of the accumulated xenobiotics and where toxic effects can be expected. Moreover, liver is the main and important detoxifying organ and is essential for both the metabolism and the excretion of xenobiotics in the body (van Dyk *et al.*, 2007), and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of biological endpoints of xenobiotic exposure (Stentiford *et al.*, 2003, Feist *et al.*, 2004 and ICES, 2006) causing histological changes in the liver. Therefore, the histological investigation may therefore produce meaningful results (van Dyk *et al.*, 2007).

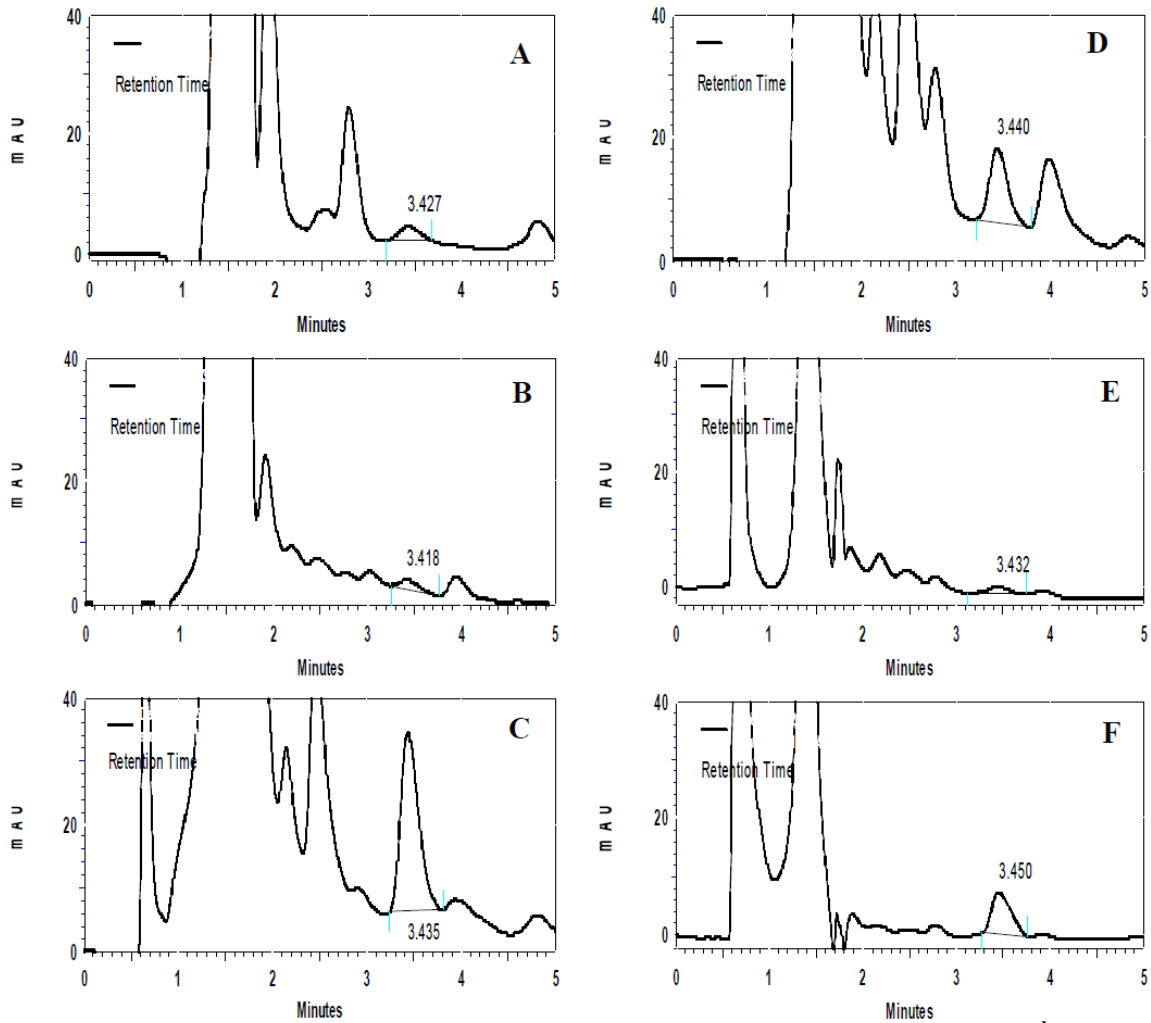


Figure 2: HPLC chromatograms of FFC Treated Broiler Chickens. A: muscle tissue (3rd day post administration, 30 mg/kg bw). B: muscle tissue (7th day post administration, 60 mg/kg bw). C: liver tissue (3rd day post administration, 60 mg/kg bw). D: liver tissue (7th day post administration, 60 mg/kg bw). E: serum (3rd day post administration, 30 mg/kg bw). F: serum (3rd day post administration, 60 mg/kg bw).

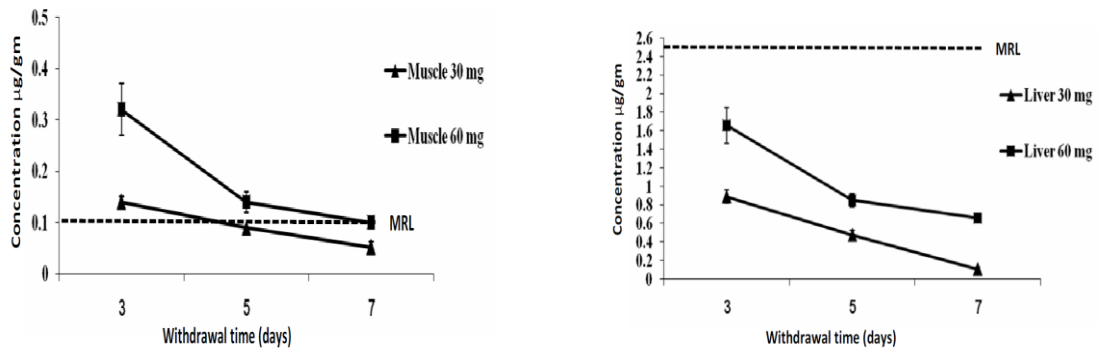


Figure 3: Residue Withdrawal Curve for Florfenicol in Muscle and Liver tissues of Broiler Chickens in relation to MRLs (100 and 2500 µg/kg, respectively) Following Oral Administration of 30 mg/kg bw and 60 mg/kg bw for 5 Successive Days.

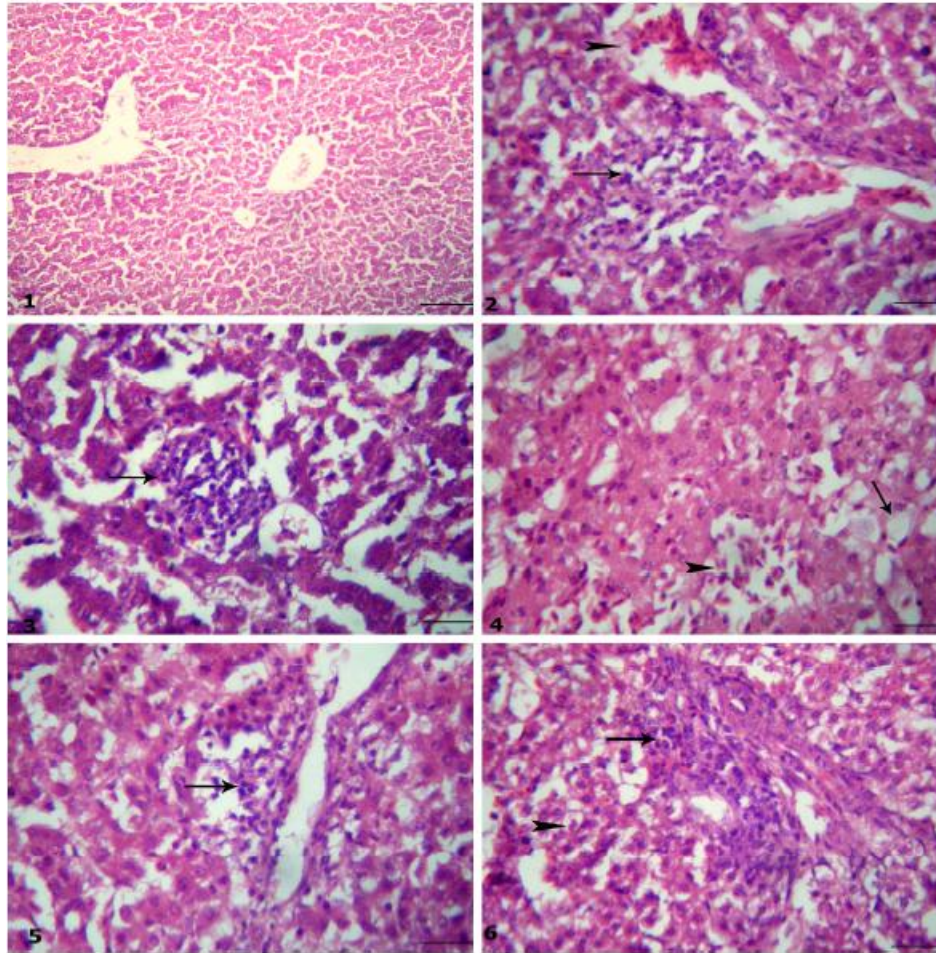


Plate (A): 1. Liver section of control group showing normal hepatocytes and sinusoidal architecture (control group). 2-6; Liver section of 30 mg/kg bw treated group showing 2. Portal area with congestion (arrowhead) and round cells infiltration (arrow) 3rd day post treatment. 3. Few interstitial lymphocytic aggregation 3rd day post treatment (arrow). 4. Mild fatty change (arrow) and extravasated erythrocytes among the hepatocytes (arrowhead) 3rd day post treatment. 5. Few round cells in the portal areas 5th day post treatment (arrow). 6. Fibroblasts proliferation and lymphocytes and few eosinophils infiltration (arrow) besides vacuolations (arrowhead) 7th day post treatment. HE (Bar = 100 μ m).

The histopathological investigation of liver tissue of control group showed normal hepatocytes and sinusoidal architecture without any degeneration or necrosis (**plate A, 1**). The liver of 30 mg/kg bw treated group showed congestion of blood vessels and round cells infiltration in the portal areas, 3rd day post administration (**plate A, 2**). Few interstitial lymphocytic aggregations (**plate A, 3**) were visualized besides mild fatty change, hydropic degeneration and extravasated erythrocytes

among the degenerated hepatocytes (**plate A, 4**). The aforementioned lesions were mostly reversible and gradually restored to the normal. Focal areas of vacuolar and hydropic degeneration were detected, 5th day post administration. Few round cells were also noticed in the portal areas (**plate A, 5**). Fibroblasts proliferation and lymphocytes and few eosinophils infiltrations (**plate A, 6**) were seen besides few vacuolated hepatocytes, 7th day post administration.

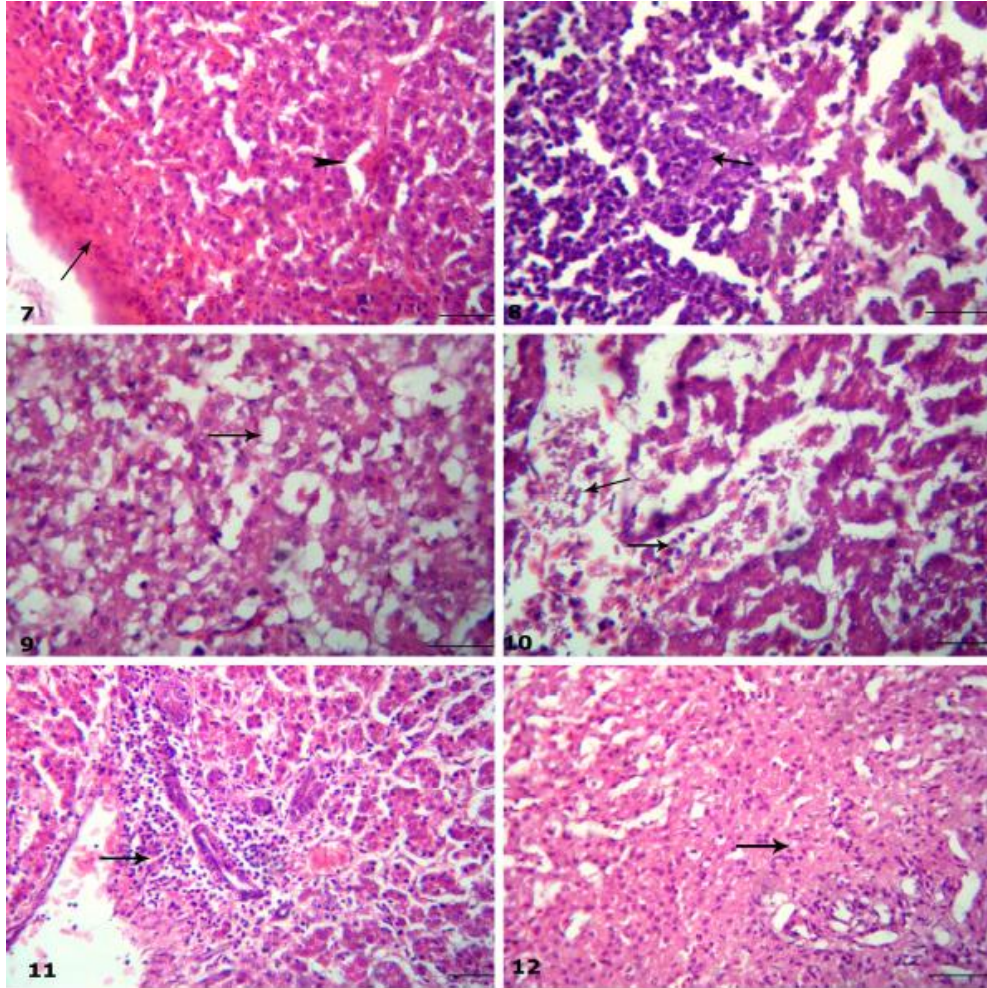


Plate (B): Liver section of 60 mg/kg bw treated group showing 7: Subcapsular coagulative necrosis (arrow) and sinusoidal congestion (arrowhead) 3rd day post treatment 8: Focal replacement of hepatic parenchyma with lymphocytes aggregation 3rd day post treatment (arrow). 9: Diffuse vacuolations of hepatic cells 5th day post treatment (arrow). 10: Irregular area of destruction and necrosis of hepatic cells 5th day post treatment (arrows). 11: Portal area with hyperplasia in the lining epithelium of bile ducts and round cells infiltrations 7th day post treatment (arrow). 12: Mild portal fibrosis infiltrated with few lymphocytes 7th day post treatment (arrow). HE (Bar = 100 µm)

In 60 mg/kg bw treated group, The liver revealed more serious effect than that in the therapeutic dose. It showed subcapsular coagulative necrosis and sinusoidal congestion (**plate B, 7**), 3rd day post administration. Focal replacement of the hepatic parenchyma with lymphocytes aggregations was observed (**plate B, 8**). Diffuse vacuolations of hepatic cells were noticed, 5th day post administration (**plate B, 9**) besides irregular areas of destruction and necroses of hepatic cells (**plate B, 10**). Sometimes, the portal areas showed hyperplasia in the lining epithelium of bile ducts and round cells infiltrations 7th day post administration

(**plate B, 11**). Mild portal fibrosis infiltrated with few lymphocytes was also reported (**plate B, 12**). This serious histopathological alteration may be explained the prolonged residual withdrawal time in this group which reflect metabolism disturbance. Microscopical examination of liver in previous studies revealed vacuolar degeneration of the hepatic parenchyma in Nile Tilapia fish (**Zaki et al., 2011**) and hypertrophy of the hepatocytes in pacu (**Patricia Carraschi et al., 2012**).

In conclusion, our results underscored the importance of monitoring drug to provide better insight for food safety issues concerning

drug residues and preventing the presence of prohibited substances in food, it should be noted that the most consumed edible tissues (muscles and liver) could have significantly different residue concentrations. Based on the MRLs, FFC should deplete from broiler chicken at least 5 days before marketing to ensure that it is eliminated from the muscle tissue following dosing to be safe for human consumption.

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