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Bioequivalence of three florfenicol preparations in broilers

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ABSTRACT

This study was aimed to determine the bioequivalence of three different preparations of florfenicol using non-drugged broiler chickens. A total of 28 broiler chickens aging 30-day were divided into four equal groups; these were Group I, II, III, and IV. The birds of Group I (for effective substance) were given intravenous (i.v.) administration of florfenicol dosed at 40 mg/kg body weight (b.wt.). The birds of Group II (for reference drug), Group III (for test-1 drug), and Group IV (for test-2 drug) received florfenicol preparations with water (dosed at 40 mg/kg b.wt.) through intracrop administration. Blood samples were collected periodically from the birds of all four groups, and blood plasma was separated. Levels of florfenicol and its metabolite (florfenicol amine) in the plasma were measured by High Performance Liquid Chromatography (HPLC). In this study, the limit of detection (LOD) for florfenicol and florfenicol amine were recorded as 0.017 and 0.78 µg/mL, respectively. On the other hand, the recovery of florfenicol and florfenicol amine were 83.4-84.6 and 82.2-83.8%, respectively. Based on the values of area under the curve (AUC), maximum concentration (C_{max}), and time to maximum concentration (T_{max}), test-1 drug was found to be acceptable, whereas test-2 drug was remained below the acceptable limits (80-125%) of AUC and C_{max}. Thus, it was concluded that test-1 drug was bioequivalent as compared to the reference drug.

Keywords

Bioequivalence, Broiler chicken, Florfenicol, Florfenicol amine, Pharmacokinetic

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INTRODUCTION

Bioequivalence studies could reveal the fact that two or more drug preparations having similar formulations could be replaced by one another (the equivalent drug). When two different drug preparations will form similar plasma densities, any one the preparations could be preferred to use (Liu et al., 2003; Yilmaz, 2006; Altintas and Eşsiz, 2008).

The benefits of doing bioequivalence tests for drug preparations which will be used for treatment in veterinary medicine might be used for the patient, the veterinarian, in the producer farm and for public health. The treatment of any illness depends on accurate diagnosis and use of suitable drug with proper dosage. Although the veterinarians used drugs for treatment purpose, without revealing out the proper drug effect, consumers' rights could not be protected in any occasion. The bioequivalence tests of drugs could be done to ensure effective and safe drug for the consumers. This will save the consumers from risks, and protecting them from unexpected problems that may arise from the use of unsafe drugs (Altintas and Yarsan, 2009; Sarica and Liman, 2007).

The fenicol group of antibiotics are one of the widely used drugs which are preferred to treat illnesses realting to digestive and respiratory systems. Nowadays, this group of anitbiotics are used in veterinary medicine effectively, especially in poultry,

against both Gram-positive and Gram-negative bacteria like *Corynebacterium pyogenes*, *Streptococcus* spp., *Staphylococcus* spp., *Clostridium* spp., *Escherichia coli*, *Pasteurella haemolytica*, *Haemophilus somnus*, and *Actinobacillus pleuropneumoniae*. Fenicol group of antibiotics includes several commonly used drugs like chloramphenicol tiamfenicol, azidamfenicol, and florfenicol. In recent times, use of florfenicol has been increased because of its safety in nature as compared to chloramphenicol (Kaya, 2007). However, very few bioequivalence studies in veterinary medicine have been reported on this drug. The aim of this study was to evaluate the bioequivalence of three different florfenicol preparations which were authorized to be used in drinking water, and to assess their suitability of using for clinical treatment purposes.

MATERIALS AND METHODS

Reagents and instruments: All reagents and solvents were of analytical grade. The reagents used in this study were: florfenicol standard (Fluka, Sigma-Aldrich, St. Louis, MO), florfenicol amine standard (TRC, Ontario, Canada), chloramphenicol standard (Sigma-Aldrich, St. Louis, MO), ethyl acetate (Sigma-Aldrich, St. Louis, MO), acetonitrile (Merck, Darmstadt, Germany), and polyethylene glycol (PEG, Sigma-Aldrich, St. Louis, MO). Ultra-pure water was obtained from a Millipore system (Millipore, Molfheim, France). Reference drug was florfenicol (300 mg/mL; 200 mL/bottle) for oral administration. Similarly, both test-1 and test-2 drugs contained florfenicol (300 mg/mL) supplied in 1 L bottle for oral administration.

Animals: Animal experiments were performed as per the guidelines as set by the Ethical Committee of Ankara University (Report No: 2010-53-264, Ankara, Turkey). Twenty eight male, Ross 308 strain, new-born chicks (0-day-old) were obtained from Bil-Yem Food Industry Limited Firm, Ankara. The chicks were kept for 7 days in separate pens on floor at 30±1^{0}C. Then the temperature was gradually reduced to 25±1^{0}C by 21-day of age. During the experiments, the chicks were given feed that was free from any drug or dirty remnants. All the chicks were accessed to water and feed *ad libitum* until 30-day of age. Light and temperature were controlled. The proportions of different nutritional values in the feed were as follow: protein 23%, metabolic energy 3,100 Kcal/kg, raw ash 8%, and raw cellulose 6%. At 30-day, the chickens were divided into four equal groups with 7 birds in each (Table 1); the groups were termed as Group 1, Group II, Group III and Group IV. Each bird was weighing approximately 2 kg as whole.

Experimental design: Blood samples were collected from the chickens of all groups before administration of drug. The blood samples were used for recovery works. The birds of Group I (for effective substance) were given a single i.v. (*Vena ulnaris superficialis*) administration of florfenicol drug dosed at 40 mg/kg b.wt., and plasma disposition of florfenicol or its metabolites were measured. The birds of Group II (for reference drug), Group III (for test-1 drug) and Group IV (for test-2 drug) were administered florfenicol preparations (dosed at 40 mg/kg b.wt.) orally directly into the crop using a thin plastic tube attached to a syringe. Food supply was withheld 12 h before oral administration of drug. Following the administration of drug, blood samples (2 mL) were collected from the birds of Group I, at intervals of 5 min (0.08 h), 10 min (0.16 h), 20 min (0.33 h), 30 min (0.5 h), 45 min (0.75 h), 1 h, 1.5 h, 2 h, 4 h, 8 h, 12 h, 18 h and 24 h. From the birds of Group II, Group III and Group IV, blood samples were collected at intervals of 10 min (0.16 h), 20 min (0.33 h), 30 min (0.5 h), 45 min (0.75 h), 1 h, 1.5 h, 2 h, 4 h, 8 h, 12 h, 18 h, 24 h and 36 h. Plasma was separated from blood by centrifugation at 500xg for 10 min, and was stored at –18^{0}C until use.

Before starting extraction procedure for High Performance Liquid Chromatography (HPLC) analysis, an internal standard solution (chloramphenicol) was added to plasma to yield a final concentration of 2 µg/mL. Extraction of florfenicol, its metabolite (florfenicol amine), and the internal standard were done following the methods described by Shen et al. (2002). In brief, 1 mL of 0.1 M phosphate buffer (pH 7.0) was added to 1 mL of plasma, and was mixed properly by vortexing for 1 min. Then, 4 mL ethyl acetate was added to the mixture, and was mixed again thoroughly. The supernatant was separated into another tube, and the same procedure was repeated to maximize the extraction. The supernatant was kept at 45^{o}C in an evaporator until it dried. The remnant was dissolved in 1 mL mobile phase, and was centrifuged at 18,000xg for 15 min. The clear parts were collected in vials, and were placed in HPLC. The auto sampler was set to take 20 µL from each sample. Considering the data obtained from the HPLC, the quantities of florfenicol and florfenicol amine in the plasma were calculated in µL/mL for each sample with the help of the standard curve that was prepared previously.

Calibration curves were obtained by calculating the ratios of the areas of florfenicol to that of chloramphenicol and plotting them against the corresponding concentration of florfenicol spiked for chicken plasma. The HPLC method for florfenicol in

chicken plasma was validated by assessing extraction efficiency and inter- and intra-day reproducibility at the concentrations of 0.2604, 0.78125, 3.125, 6.25, 12.5 and 50 µg/mL. The HPLC apparatus (Shimadzu LC-20A, HPLC, Shimadzu, Tokyo, Japan) set with a photo diode-array detector and inertsil ODS-3 column (250mm X 4,6mm X 5µm) (GL Sciences, Eindhoven, The Netherlands) was used for the measurement of florfenicol and/or florfenicol amine. The conditions of HPLC apparatus were: column heat 30°C, mobile phase; acetonitrile:water (27:73; v/v), 223 nm wavelength, and 0.6 mL/min flow speed.

Pharmacokinetics and statistical calculations: The three drugs were determined as suitable for the external model of the body movement based on two parts of plasma concentration-time curve and the calculations done by considering the Pharmacokinetic Calculation (PKCALC) (Shumaker, 1986) program. "SPSS 15.0 for Windows" statistic packet program was used for the statistical analysis (Altintas and Yarsan, 2009). The data were explained by means of the arithmetic mean±SD at the maximum and minimum values. The variance analysis with one direction (ANOVA) was applied to pharmacokinetic data and the significance of the differences between the groups was determined by the Duncan test (Altintas and Yarsan, 2009). The time curve was drawn considering the drug density measured in the plasma samples. According to the curve, the area under the curve (AUC), maximum concentration (C_{max}), and time to maximum concentration (T_{max}) were calculated. The bioequivalence between the drugs were evaluated by using *EquivTest* statistical program (Equivtest, 2011).

RESULTS AND DISCUSSION

In HPLC, florfenicol gave a peak at 21.69 min, florfenicol amine gave a peak at 9.72 min, and the internal standard gave a peak at 25.55 min. Standard curve was drawn with the help of the peak areas obtained from the standards and the equation of the linear curve obtained (Figure 1). The quantities of florfenicol and florfenicol amine included in the plasma was determined by placing the peak areas obtained from the plasma in the equation calculated from the standards and the data were evaluated as µg/mL plasma. The sensitivities of the method (LOD) were 0.017 µg/mL for florfenicol, and 0.78 µg/mL for florfenicol amine. On the other hand, recoveries were 83.4-84.6% for florfenicol and 82.2-83.8% for florfenicol amine.

After i.v. (florfenicol active substance) and intracrop administrations (reference, Test 1 and Test 2 drug), it was revealed that the movements of florfenicol (r^2:0.998) and florfenicol amine (r^2:0.997) in the body were suitable for the external model with two parts by considering the drug concentration-time curve (Figure 2 and Figure 3). The bioequivalence of reference, test-1 and test-2 drugs were presented in Table 2.

In veterinary medicine, especially in poultry, fenicol antibiotics are used as one of the most effective antibiotics. Use of florfenicol, an important representative of fenicol antibiotics, has recently been increased because of its safety in nature as compared to chloramphenicol. In this study, the limit of detection (LOD) for florfenicol and florfenicol amine were recorded as 0.017 and 0.78 µg/mL, respectively. These findings were in support of the findings of Kim et al. (2011) who reported the LOD as 0.015 µg/mL. However, the sensitivity of our method was less than the reports of Switala et al. (2007) and Vue et al. (2002), who reported the LOD as 0.007 and 0.0045-0.0087 µg/mL, respectively). However, our findings were higher than the results described by Koc et al. (2009), Gaikowski et al. (2010), Jianzhong et al. (2004) and Kowalski et al. (2005). These differences on the sensitivity limitations might be due to differences in working conditions, the extraction works, the analysis methods, and the characteristics of the HPLC apparatus.

The proportion of the recovery calculated for florfenicol and its metabolite (florfenicol amine) were 83.4-84.6 and 82.2-83.8%, respectively. The value found in our study for florfenicol was similar to the results reported by Koc et al. (2009), who found the value as 85±6.33%. On the other hand, our results were lower than the results of Park et al. (2007), Wrzesinski et al. (2006), Anadon et al. (2008), Shen et al. (2003) and Lane et al. (2004). The data for the proportion of the recovery calculated in this study was proved to be safe.

In this study, the bioequivalence of the three drugs including florfenicol active substance using broilers were evaluated for the first time. As a general rule, for the evaluation of bioequivalence and comparison of reference and test drugs, pharmacokinetic variations like AUC, C_{max} and T_{max} should be 90% confidence interval (CI) and within 80-125% limit. As the value of C_{max} presents great variations during the time of the example, the CI might be accepted as 70-143% limitations.

Table 1. The groups used to determine the density of florfenicol in plasma.

Group	Florfenicol Dose	Application route	Substance
Group I	40 mg/kg b.wt.	i.v. (*Vena ulnaris superficialis*)	Effective substance
Group II	40 mg/kg b.wt.	intracrop (oral)	Reference drug
Group III	40 mg/kg b.wt.	intracrop (oral)	Test-1 drug
Group IV	40 mg/kg b.wt.	intracrop (oral)	Test-2 drug

Table 2. Bioequivalence reference, test-1 and test-2 drugs for pharmacokinetic parameters.

Parameters	Drugs	AUC (µg.h/mL)	C_{max} (µg/mL)	T_{max} (h)
Florfenicol	Reference drug	60.94	8.74	1.5
	Test-1 drug	51.95	7.46	1.5
	Test-2 drug	43.90	6.30	1.5
Bioequivalence	Test-1 drug	85.24%	85.35%	100%
	Test-2 drug	72.03%	72.08%	100%
Acceptable limits		80-125%		

AUC = Area under the concentration; C_{max} = Maximum concentration; T_{max} = Time to maximum concentration.

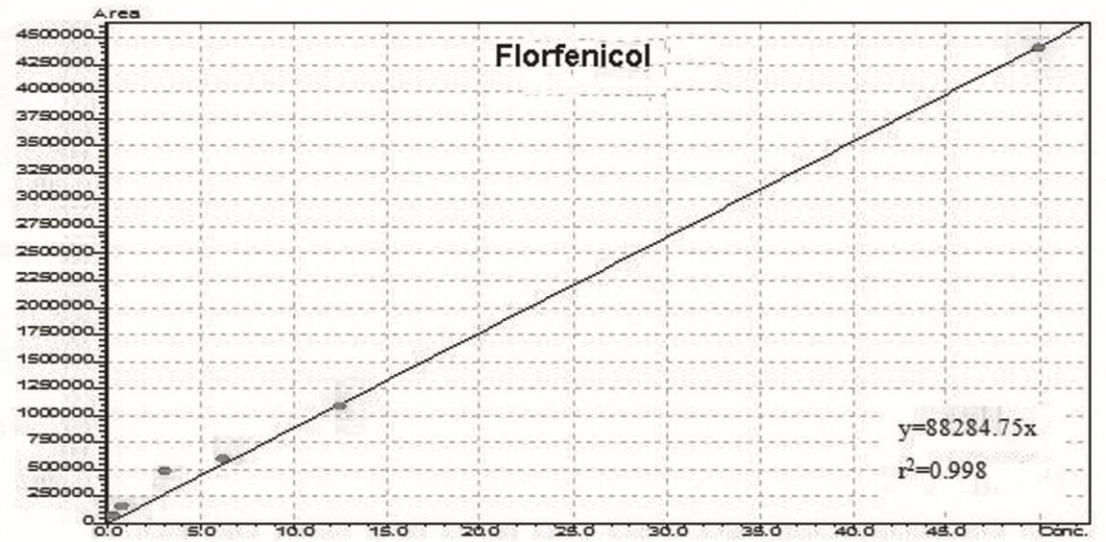

Figure 1. The standard curve of the concentrations of florfenicol prepared using peak areas obtained in HPLC.

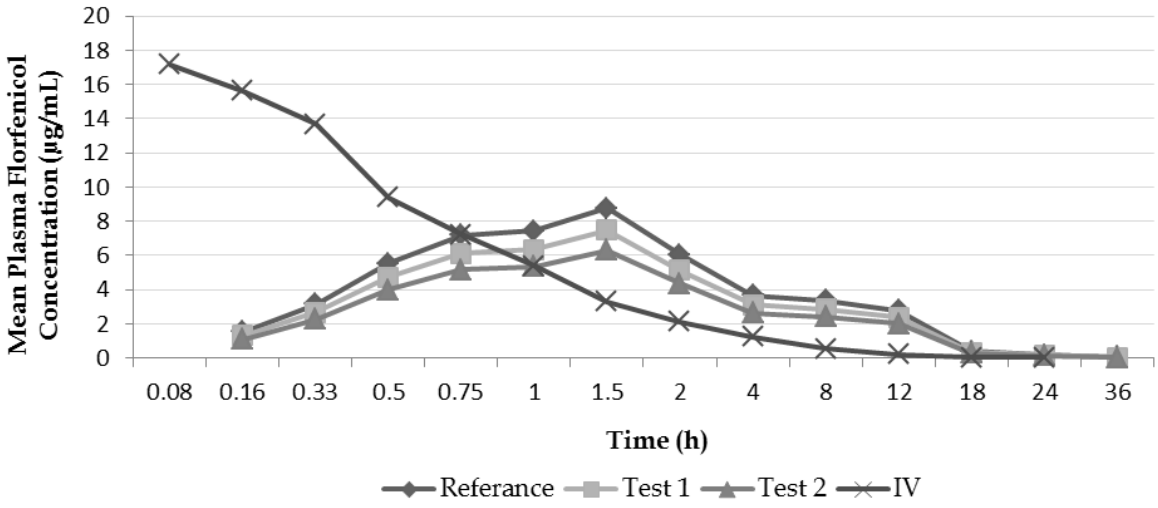

Figure 2. Florfenicol semi-logaritmic plasma concentration-time curve after i.v. (florfenicol active substance) and intracrop (reference, test-1 and test-2 drug) administrations. Values are arithmetic means.

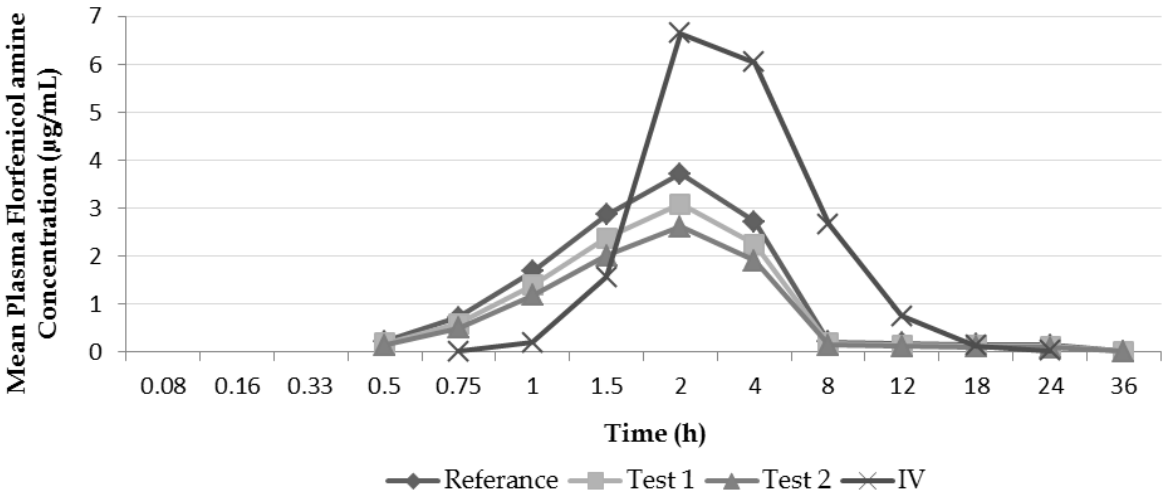

Figure 3. Florfenicol amine semi logaritmic plasma concentration-time curve after intravenous (florfenicol active substance) and intracrop (reference, test-1, test-2 drug) administrations. Values are arithmetic mean.

The values obtained with the division of the AUC values of test-1 and test-2 drugs (51.95 µg.h/mL; 43.90 µg.h/mL, respectively) into the value of the reference drug (60.94 µg.h/mL) were 0.85 and 0.72 respectively. The values obtained with the division of the C_{max} values of test-1 and test-2 drugs (7.46 µg/mL; 6.30 µg/mL) into the value of reference drug (8.74 µg/mL) were 0.85 and 0.72, respectively (Table 2). It was determined that the AUC, C_{max} and T_{max} values of the test-1 and reference drugs were within 80-125% limit for bioequivalence. These data showed that test-1 drug is in bioequivalence with the reference drug and that they could be interchanged with each other. It was determined that the AUC and C_{max} values of the test-2 drug and the reference drug were not within 80-125% limit for bioequivalence. This result showed that test-2 drug was not bioequivalent with the reference drug and that they cannot be replaced with each other.

CONCLUSIONS

Based on comparison of the AUC, C_{max} and T_{max} values for reference and test drugs, it can be concluded that test-1 drug was bioequivalent. On the other hand, test-2 drug was not bioequivalent in terms of AUC and C_{max} values.

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