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Effects of feed intake and water hardness on fluralaner pharmacokinetics in layer chickens

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ABSTRACT

Background: Fluralaner is a novel drug belonging to the isoxazoline class that acts on external parasites of domestic animals. It is used systemically via drinking water, especially against red poultry mite in layer chickens. Fluralaner is frequently used in layers infected with *D. gallinae*. However, no study to date has investigated the effects of feed intake and water hardness.
Objectives: This study aimed to investigate the effects of variable water hardness and feed intake on the pharmacokinetic profile of fluralaner.
Methods: Layer chickens were divided into four groups (n = 8): fed + purified water (Group 1), feed restricted + purified water (Group 2), feed restricted + hard water (Group 3), and feed restricted + soft water (Group 4). After administering a single dose of the drug with drinking water, the blood samples were collected for 21 days. Fluralaner concentrations in plasma samples were determined by liquid chromatography/tandem mass spectrometry. The maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (t_{max}), area under the concentration–time curve values ($AUC_{0\text{-}21d}$), half-life ($t_{1/2}$), and other pharmacokinetic parameters were calculated.
Results: Although the highest maximum plasma concentration (C_{max}) was determined in Group 1 (fed + purified water), no statistically significant difference was found in the C_{max}, t_{max}, $t_{1/2}$, $MRT_{0\text{-}inf\_obs}$, Vz/F_{obs}, and $Cl/F_{\_obs}$ parameters between the experimental groups.
Conclusions: It was concluded that the feed intake or water hardness did not change the pharmacokinetic profile of fluralaner in layer chickens. Therefore, fluralaner could be used before or after feeding with the varying water hardness in poultry industry.

Keywords: Isoxazoles/pharmacokinetics; fluralaner; antiparasitic agents/pharmacokinetics; area under curve; food-drug interactions; poultry; fasting

INTRODUCTION

The poultry red mite (*Dermanyssus gallinae*) is recognized in many countries as a blood-sucking ectoparasite that affects the economy of the egg-laying industry [1]. The percentage of farms infested with *D. gallinae* in Europe was reported as 83% [2]. *D. gallinae* causes anemia, stress to birds, suppression of the immune system, and weight loss in addition to a decline in the quality of egg production in layer chickens, thereby causing economic losses [3,4]. Also,

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Conflict of Interest
The authors declare no conflicts of interest.

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mites are capable of spreading zoonotic diseases and impacting animal health, welfare, and public health [5].

The development of resistance in mites due to long-term and repeated use of insecticides is a commonly observed condition. *D. gallinae* field populations were reported to have 86% resistance to the highest concentrations of carbaryl and 42% resistance to permethrin [6]. The discovery of new drugs has become an important issue due to the high resistance to classical drugs.

Isoxazoline-class drugs were discovered after 2010 as a class of ectoparasiticide. From 2013, afoxalaner, fluralaner, sarolaner, and lotilaner active ingredients have been used in the veterinary field [7]. Fluralaner (carbamoyl benzamide phenyl isoxazoline) is a newly discovered drug active substance of the isoxazoline class that acts on the external parasites of domestic animals.

Fluralaner kills blood-sucking mites in chickens for 15 days after the first administration and stops the egg production of female mites [8]. Fluralaner is used orally with drinking water in layer chickens. The oral bioavailability has been reported to be 91%. Fluralaner binds highly to plasma proteins and has a large volume of distribution in the body. The half-life after oral administration is about 5 days [8-11]. Adequate information on the effects of feed intake on the pharmacokinetics of fluralaner in poultry is not available. Feed intake can affect the distribution of the drug formulation and the dissolution of the drug in the gastrointestinal tract.

Also, water hardness is a critical physiochemical factor in the administration of drugs with drinking water [12]. Some antibiotics can interact with cations to form stable complexes. The increase in the hardness of drinking water reduces the bioavailability of enrofloxacin [13,14]. In the light of this information, the evaluation of the relationship between the pharmacokinetics of a drug and water hardness is an important issue. Hence, this study aimed to determine the effect of feed intake and water hardness on the pharmacokinetic profile of fluralaner in layer chickens.

MATERIALS AND METHODS

Reagent and drugs

The reference standard of fluralaner (99.83%, 864731-61-3) was purchased from MedChem Express (China). Formic acid, methanol, acetonitrile, and reserpine (43530) were obtained from Sigma–Aldrich Chemical Co (Germany). All the other chemicals used were analytical grade and obtained from commercial sources.

Experimental animals

All laying chickens (Lohmann brown) were obtained from a commercial breeding farm (Kale Poultry Inc., Turkey). The animals were housed in cages according to species requirements. Each cage had a feeder and a bell-shaped drinker. The condition of 12 h of light followed by 12 h of darkness and natural ventilation was applied. Water were supplied *ad libitum*. Birds were fed a standard layer diet (Ipek Feed and Food Inc., Turkey) throughout the study. The standard layer diet contained per kilogram 175 g of crude protein, 40 g of crude fiber, 36 g of crude fat, 150 g of crude ash, 8 g of lysine, 4 g of methionine, 40 g of calcium, 4 g of phosphorus, and 2 g of sodium. The chickens were kept under acclimatization for 4 weeks

without any drug administration. The experiments were conducted with approval from the Local Ethics Committee of Animal Experiments of Kırıkkale University (No. 53 dated 10.04.2018).

Experimental design

A total of 32 laying chickens, weighing 2–3 kg, were divided into 4 groups (n = 8): fed + purified water (Group 1), feed restricted + purified water (Group 2), feed restricted + hard water (Group 3), and feed restricted + soft water (Group 4). No feed restriction was applied in Group 1 animals. Groups 2, 3, and 4 animals were not fed 12 h before and 6 h after drug administration. Fluralaner (Exzolt, 0.5 mg/kg bw) was administered via drinking water in Group 1 and 2 animals. Fluralaner (0.5 mg/kg bw) in hard water was given to Group 3 to study the effect of water hardness on drug pharmacokinetics. Group 4 received fluralaner (0.5 mg/kg bw) in soft water by oral gavage. Soft water and hard water were given to Group 3 and 4 animals during the experiment, respectively.

The drug and drinking water mixtures were prepared fresh in accordance with the application doses and instructions reported by the European Medicines Agency (EMA) for fluralaner [8]. Soft and hard water samples were prepared from purified water (pH 6) by adding 40 mg/L calcium carbonate (pH 7.3) and 400 mg/L calcium carbonate (pH 9), respectively [15]. Soft water and hard water samples were prepared under the regulation of the EMA on the administration of drinking water and veterinary drugs [16].

The blood samples from all groups were taken in vacuum tubes with heparin 15, 30, and 45 min, 1, 3, 6, 10, 12, 24, and 36 h, and then 2, 3, 4, 5, 7, 14, and 21 days after oral administration. The sampling times were determined according to pharmacokinetic data reported to cover the absorption, distribution, and elimination periods.

Sample preparation

The blood samples were centrifuged, and the plasma was separated at 2,000 rpm for 10 min at 4°C. The plasma samples were stored in Eppendorf tubes at −20°C until analysis. The chromatographic analysis of fluralaner from plasma samples was performed according to the Kilp et al. [17] with slight modifications. For protein precipitation, 500 µL of plasma was vortexed for 30 sec after adding 50 µL of the internal standard and 450 µL of acetonitrile. Reserpine (1 mg/mL in acetonitrile) was used as the internal standard. The resultant solution samples were centrifuged at 5,000 rpm for 10 min, and the supernatants were transferred to HPLC vials and stored at −20°C until chromatographic analysis.

Determination of fluralaner

All sample vials were analyzed using liquid chromatography–mass spectrometry (Thermo Scientific Ultimate 3000 UHPLC system, Thermo Scientific TSQ Fortis Triple Quadrupole Mass Spectrometer, and Thermo Scientific Ion Max source with HESI-II probe; Thermo Scientific, USA).

The column temperature was set to 45°C, and the injection volume was 2 µL. Mobile phase A was 0.1% formic acid (in water), and Mobile Phase B was 0.1% formic acid (in methanol). A binary gradient at the flow rate of 0.3 mL/min is shown in **Table 1**.

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Table 1. UHPLC-MS/MS method gradient profile

Time (min)	Mobile phase A (vol %)	Mobile phase B (vol %)
0.0	80	20
0.2	80	20
1.0	30	70
3.0	10	90
5.0	10	90
5.1	80	20
7.0	80	20

MS/MS, tandem mass spectrometry.

Pharmacokinetic calculations and statistical analysis

The pharmacokinetic parameters were calculated using the noncompartment analysis with the validated PKSolver add-in program for Microsoft Excel. The maximum plasma concentration (C_{max}) and the time to reach maximum plasma concentration (t_{max}) were determined. The half-life ($t_{1/2}$) was calculated by linear regression of the terminal segment and using the top of the area under the curve (AUC) from time zero until the time of the last measurable concentration ($AUC_{0\text{-}t}$) was calculated using the linear trapezoidal rule. Also, the mean residual time ($MRT_{0\text{-}inf\_obs}$), volume of distribution ($Vz/F_{\_obs}$), and plasma clearance ($Cl/F_{\_obs}$) parameters were calculated.

Statistical analysis was performed using IBM SPSS Statistics version 21 (IBM Corp., USA). All pharmacokinetic parameters for fluralaner were statistically compared using one-way analysis of variance followed by the Tukey's HSD *post hoc* test. Statistical significance was assumed at p values ≤ 0.05.

RESULTS

The calibration curves were in good linearity over seven different concentrations between 10 and 2500 ng/mL, with a correlation coefficient of $r^2 = 0.9734$. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the validated linear range for fluralaner using seven concentrations: 1, 5, 10, 25, 60, 100, and 250 ng/mL. LOD was determined as 3.1 ng/mL, and LOQ as 9.6 ng/mL. The recovery of 60, 250, and 1000 ng/mL in the calibration range was determined as 109.81%, 103.41%, and 92.67%, respectively, in 10 replicates.

The intra-day precision (CV) and accuracy (RE) were estimated by analyzing five replicates containing fluralaner at three different quality control levels, that is, 0.6, 1, and 10 μg/mL. The inter-assay precision was determined by analyzing the three levels of QC samples (0.6, 1, and 10 μg/mL) on different three runs.

The intra-day accuracy (%) ranged from 102.50 to 104.50 at 0.6 μg/mL, 99.90 to 101.30 at 1 μg/mL, and 99.66 to 101.02 at 10 μg/mL. The inter-day accuracy (%) was 106.59, 99.70, and 99.83 at 0.6, 1, and 10.0 μg/mL, respectively. The intra-day precision (relative standard deviation; %RSD) was 3.53 at 0.6 μg/mL, 0.58 at 1 μg/mL, and 0.25 at 10 μg/mL. The inter-day precision (%RSD) was 7.72, 3.15, and 2.91 at 0.6, 1, and 10.0 μg/mL, respectively. The validation parameters of the method are presented in **Table 2**.

All animals were alive and healthy during the experimental period, and no adverse reactions were observed. Fluralaner was found to be similarly absorbed and reached similar C_{max} and t_{max} in both feed-restricted and fed chickens and in different strengths of water. When the fluralaner plasma

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Table 2. Plasma fluralaner validation results in LC-MS/MS analysis

Parameters	Fluralaner chromatographic conditions
Linearity range (ng/mL)	1–250
r^2 value	0.9734
LOD (ng/mL)	3.1
LOQ (ng/mL)	9.6
Intraday repeatability (%RSD) (n = 5)	
0.6 μg/mL	3.53
1 μg/mL	0.58
10 μg/mL	0.25
Interday repeatability (%RSD) (n = 5)	
0.6 μg/mL	7.72
1 μg/mL	3.15
10 μg/mL	2.91
% Recovery	
60 μg/mL	109.81
250 μg/mL	103.41
1,000 ng/mL	92.67
Retention time (min)	4.90

LC, liquid chromatography; MS/MS, tandem mass spectrometry; r^2, correlation coefficient; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

concentration curve was considered, it was found to be suitable for noncompartmental analysis. No significant difference was found in t_{max} between groups. The mean of t_{max} in all groups was 1.3 ± 0.37 days. The mean C_{max} of all groups was found to be 336.53 ± 90.76 ng/mL. No statistically significant difference was found between groups in fluralaner C_{max}. The plasma concentration/time profile of groups after fluralaner administration in plasma are shown in **Fig. 1**, and the pharmacokinetic parameters are shown in **Table 3**. The mean of $t_{1/2}$ was determined as 4 ± 0.24 days. The average $AUC_{0\text{-}21}$ in all groups was determined as 1,582.65 ± 374.14 ng/mL per day. The $AUC_{0\text{-}21}$ showed no statistically significant difference between the groups. The AUC increased in Group 1 and Group 4, but the difference did not reach statistical significance due to the wide variation of the values in the chickens.

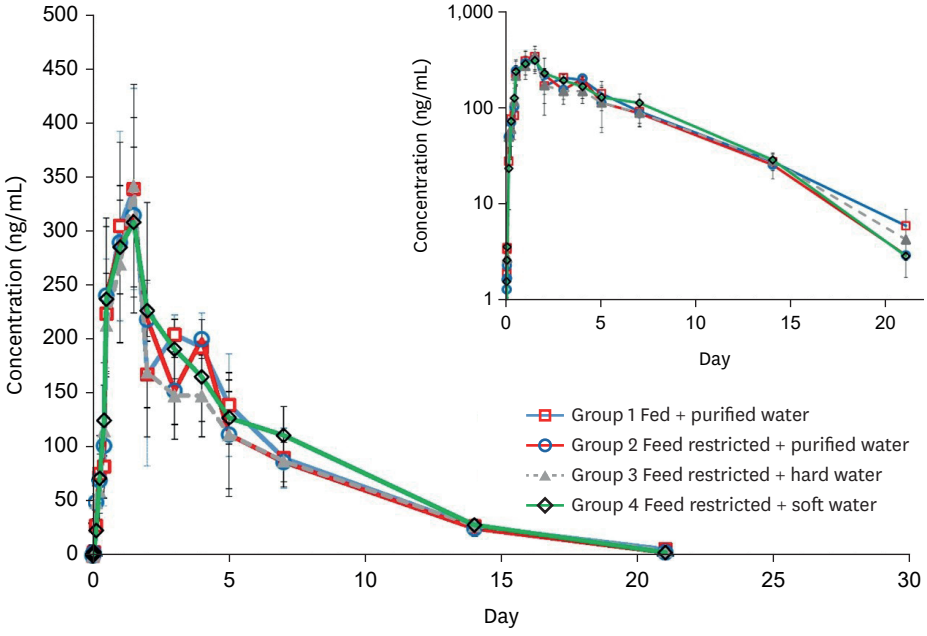

Fig. 1. Pharmacokinetic profile of fluralaner. Data are presented as means and standard deviation of the mean (n = 8).

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Table 3. Pharmacokinetic parameters of the experimental groups (n = 8)

Parameters	Unit	Group 1 Fed + purified water	Group 2 Feed restricted + purified water	Group 3 Feed restricted + hard water	Group 4 Feed restricted + soft water	Total group	*p* value[a]
Lambda z	1/day	0.17 ± 0.05	0.18 ± 0.06	0.18 ± 0.09	0.23 ± 0.07	0.19 ± 0.07	0.320
$t_{1/2}$	day	4.32 ± 1.43	4.14 ± 1.38	4.43 ± 1.66	3.12 ± 0.85	4.00 ± 1.40	0.226
t_{max}	day	1.31 ± 0.25	1.31 ± 0.37	1.43 ± 0.17	1.25 ± 0.59	1.32 ± 0.37	0.877
C_{max}	ng/mL	369.59 ± 92.42	350.34 ± 87.99	326.28 ± 103.74	299.89 ± 79.44	336.53 ± 90.76	0.467
$AUC_{0\text{-}21d}$	ng/mL* day	1,707.07 ± 424.84	1,507.82 ± 317.89	1,343.58 ± 428.91	1,772.12 ± 154.70	1,582.65 ± 374.14	0.081
$AUC_{0\text{-}inf\ obs}$	ng/mL* day	1,993.82 ± 302.06	1,731.11 ± 280.31	1,594.82 ± 413.04	1,825.66 ± 138.31	1,786.35 ± 320.85	0.080
$MRT_{0\text{-}inf\_obs}$	day	6.17 ± 2.06	6.12 ± 1.40	6.20 ± 1.62	5.57 ± 0.54	6.02 ± 1.46	0.814
Vz/F_{obs}	(mg/kg)/(ng/mL)	0.015 ± 0.005	0.017 ± 0.007	0.020 ± 0.008	0.123 ± 0.003	0.016 ± 0.006	0.080
Cl/F_{obs}	(mg/kg)/(ng/mL)/day	0.002 ± 0.0003	0.002 ± 0.0004	0.003 ± 0.001	0.002 ± 0.0002	0.002 ± 0.0006	0.079

Data are presented as mean ± SD.
$t_{1/2}$, half-life; t_{max}, time to reach maximum plasma concentration; C_{max}, maximum plasma concentration; $AUC_{0\text{-}21d}$, area under the concentration–time curve between zero and the last sampling point; $AUC_{0\text{-}inf\ obs}$, area under the concentration-time curve from zero up to infinity; $MRT_{0\text{-}inf\_obs}$, mean residence time from zero up to infinity; Vz/F_{obs}, apparent volume of distribution during terminal phase after oral administration; Cl/F_{obs}, Apparent total plasma or serum clearance of drug after oral administration; ANOVA, analysis of variance; SD, standard d.
[a]One-way ANOVA.

Despite a difference between the C_{max}, t_{max}, $t_{1/2}$, $MRT_{0\text{-}inf\_obs}$, Vz/F_{obs}, and $Cl/F_{\_obs}$ values between the groups, the difference in all pharmacokinetic data between the groups was not statistically significant.

DISCUSSION

Feed intake is an important factor that directly affects the physiology of the digestive system. Hence, the administration of drugs to fed animals may cause differences in the pharmacokinetic profile of drugs [18]. Feed-drug interaction may occur directly through binding of the drug with feed components or may occur indirectly as a result of changes in gastrointestinal physiology (motility, small intestinal transit times, pH, or hepatic blood flow). Ingested food may enhance drug absorption by increasing the solubility of active pharmaceutical ingredients, delaying gastric emptying, or stimulating bile flow. On the contrary, the drug absorption may be decreased by food through the stimulation of gastric acid secretion and intestinal motility, or directly interaction with the active ingredient of the drug. Food can increase drug absorption and exposure by increasing drug dissolution, solubility, and hepatic blood flow, delaying gastric emptying, or decreasing drug absorption by interacting with the drug or stimulating gastric acid secretion [19]. In the light of this information, feeding time is considered as an important factor affecting the pharmacokinetic profile of the drugs.

To date, the interaction between the presence of feed in the digestive system and drugs has been confirmed by many scientific reports [20-22]. Similar to drug–drug interaction, food–drug interaction is important for drugs with a narrow therapeutic index, which undergoes biotransformation, are highly bound to plasma proteins and transported by membrane transport proteins, and have affinity to specific target points [23]. In this context, determination of the interaction between feed and fluralaner is considered valuable for research due to the high plasma protein binding (99.9%) of isoxazolines and their long half-lives [7]. Despite no comprehensive research on the effect of feed intake on the pharmacokinetics of fluralaner in poultry, the effect of concurrent feeding on fluralaner pharmacokinetics has been investigated on feed-restricted and fed dogs [24]. In this study, Walther et al. [24] reported that the co-administration of drug with feed increased the C_{max} (approximately 2.1-fold) and significantly increased the bioavailability. In another study

investigating the effect of feeding on the pharmacokinetics of lotilaner, an isoxazoline ectoparasitic, lower bioavailability was reported in feed-restricted dogs [25].

Similar to these results in dogs, our data demonstrated that the oral administration of fluralaner with feed increased plasma drug concentration by 5.49%, but this increase was not considered statistically significant. Higher C_{max} values for fed dogs reported by Walther et al. [24] might be the result of different anatomical structure of the gastrointestinal tract in avian species or the administration of fluralaner in different pharmaceutical forms.

Generally, the nutritional factors in the feed can chemically bind to drugs and thus alter the pharmacokinetics of the drug by making insoluble salts that are not easily absorbed. On the contrary, a fat-rich diet can increase the oral absorption of lipid-soluble drugs by increasing their solubility [26]. Considering the fact that fluralaner is highly lipid-soluble as a common feature of isoxazoline derivatives [7], the higher C_{max} value in the fed chickens than in feed-restricted chickens can be explained by the higher solubility of the drug in the gastrointestinal tract. In addition, C_{max} values determined for different experimental groups (299.8–369.59 ng/mL) were found to be quite close to the reported value for fluralaner (Exzolt) administered with drinking water in the EMA technical report (323.7 ng/mL) [11].

In the evaluation of feed–drug interactions, t_{max} is also an important parameter. In the avian species, the passage of ingesta from the crop may delay oral drug absorption [27]. In addition, the chemical and physical properties of the drugs are important factors for their interaction potential with feed [27]. Especially acid- or base-soluble drugs may precipitate in the crop, and as a result, oral absorption may be delayed [28]. In the study conducted by Laczay et al. [29] the pharmacokinetic profile of doxycycline was investigated in fed and feed-restricted chickens. A prolonged t_{max} was observed in the fed group due to the presence of feed in the intestinal tract. However, in our study, the plasma concentration curve started to increase without a lag phase. We suggested that fluralaner was not precipitated in the crop when applied via drinking water and reached the intestine in a few minutes directly through the crop and gizzard. Our results were consistent with the findings of Walther et al. [24], who revealed that the time to reach maximum plasma concentration of fluralaner was quite similar in fed and feed-restricted dogs. On the contrary, in our study, t_{max} values ranged between 30 and 34 h, and the mean t_{max} all groups was determined as 31.68 h. These results were very close to the t_{max} value reported in the EMEA report for fluralaner (36 h) when administered with drinking water [10].

In our study, although the $AUC_{0\text{-}inf}$ value (1,993.82 ± 302.06 ng/mL per day) of fluralaner was 10%–25% higher fed group than in the other groups, no statistically significant difference was observed. Despite the apparent differences between the groups, the lack of statistical significance might be a result of the high variation within the groups. In addition to the AUC data, we observed a high variation in the C_{max} parameter within the groups. This situation might be explained by the fact that the stress during blood sampling caused fluctuations in the biochemical parameters of chicken, thus affecting individual pharmacokinetic values [30,31]. It has also been suggested that a much larger sample size may be required to detect statistical differences in studies evaluating the effects of feed or water intake on the pharmacokinetics of drugs [15]. The average $t_{1/2}$ was determined as 4.00 ± 1.40 days in the groups, similar to that stated in the EMEA report (about 5 days after oral administration) [8,32]. Our findings demonstrated that the feed intake and water hardness did not affect the metabolism and excretion rate of the drug, considering no significant differences in $t_{1/2}$ between the groups.

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In conclusion, the co-administration of a drug with feed may affect pharmacokinetic profile of drugs. In addition, based on the previous reports, it is expected that the interaction of drug(s) with feed may significantly alter the drug response. However, our results indicated that the feed intake status and water hardness had no significant effect on the pharmacokinetic profile of fluralaner in laying chickens and therefore fluralaner could be used before or after feeding with the varying water hardness in poultry industry. As a result of the fact that the pharmacokinetic profile did not change depending on the feed intake and water hardness, this may provide an advantage in terms of ease of use in collective treatments and prophylactic use in intensive or extensive poultry farming. In further studies, it is recommended to examine the fluralaner accumulation in parasites and ecosystem and modify the use accordingly.

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