



Safety evaluation of orally administered afoxolaner in 8-week-old dogs



Marlene Drag*, Judith Saik, Jay Harriman, Diane Larsen

Merial Limited, 3239 Satellite Boulevard, Duluth, GA 30096-4640, USA

ARTICLE INFO

Keywords:
Afoxolaner
Oral treatment
Dogs
Safety

ABSTRACT

The safety profile of afoxolaner, a new isoxazoline molecule, was evaluated following the regulatory requirements when administered six times orally in a soft chewable formulation at a dose of at least 1×, 3× or 5× the maximum exposure dose (6.3 mg/kg) in 8-week-old Beagle dogs. Thirty-two healthy puppies (16 males and 16 females) were enrolled and allocated randomly to one of four treatment groups. Treatments were administered at three, one-month dose intervals (Days 0, 28 and 56) followed by three, 2-week dose intervals (Days 84, 98 and 112). The study ended at Day 126. The groups were: Group 1: non-treated control; Group 2: afoxolaner chews administered at a dosage of at least 6.3 mg/kg (1×); Group 3: afoxolaner chews administered at a dosage of at least 18.9 mg/kg (3×); and Group 4: afoxolaner chews administered at a dosage of at least 31.5 mg/kg (5×). All dogs were examined for general health twice a day beginning on at least Day-14. Physical examinations, and blood collections for clinical pathology analysis and afoxolaner plasma concentrations, were performed throughout the study. On Day 126, 2 weeks following the last treatment, all dogs were humanely euthanized prior to the conduction of a full necropsy with tissue collection.

No afoxolaner-related changes were observed in growth, physical variables, clinical pathology variables, or tissues examined histologically. No clinically or statistically significant health abnormalities related to the administration of afoxolaner were observed. Vomiting and diarrhea were observed sporadically across all groups including the controls. The kinetics of afoxolaner plasma concentrations was linear following 6 doses of 6.3, 18.9 and 31.5 mg/kg and dose proportionality was demonstrated. There were no statistical differences ($p < 0.05$) between samples taken on Days 55 and 83 when compared to Day 27. Based upon the results of this study, afoxolaner was shown to be safe when administered repeatedly in a soft chewable formulation at up to 5× the maximum exposure dose in dogs as young as 8 weeks of age.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

1. Introduction

Advances in research for ectoparasitological control have brought new therapeutic drugs forward for clinical

usage (e.g., fipronil, imidacloprid and spinosad) (Beugnet and Franc, 2012). Afoxolaner is a compound from a new structurally unique isoxazoline class which acts as a novel and specific blocker of insect ligand-gated chloride ion channels (Shoop et al., 2014). It was formulated in a unique soft, beef-flavored chew (Nexgard®, Merial). There are four chew sizes, of respectively 0.5 g, 1.25 g, 3 g and 6 g, containing 11.3 mg, 28.3 mg, 68 mg and 136 mg of afoxolaner.

* Corresponding author. Tel.: +1 573 392 9334; fax: +1 573 642 0356.
E-mail address: Marlene.Drag@Merial.com (M. Drag).

Table 1
Description of afoxolaner treatment groups.

Treatment group (number of dogs)	Treatment (dose; route)	Treatment days
1 (8 ^a)	Control (not applicable)	
2 (8 ^a)	1 × afoxolaner (≥6.3 mg/kg; oral)	0, 28, 56, 84, 98, 112
3 (8 ^a)	3 × afoxolaner (≥18.9 mg/kg; oral)	
4 (8 ^a)	5 × afoxolaner (≥31.5 mg/kg; oral)	

^a Equal numbers of each sex were included.

They are intended for dogs weighing 2–4 kg, 4.1–10 kg, 10.1–25 kg and 25.1–50 kg, respectively. The weight bands of the various chew sizes can result in a minimum therapeutic dose of 2.5 mg/kg and a maximum exposure dose of 6.3 mg/kg body weight.

The assessment of the safety of a compound in the target species is a prerequisite for registration of veterinary products. The guidelines for target animal safety studies now require that the compound be tested using the final commercial formulation at 1, 3, and 5 times the maximum exposure dose (VICH, 2008). Oral as well as topically applied antiparasitic drugs are usually manufactured so that one size tablet/chewable or pipette can be administered to animals within a specified weight range (Blagburn et al., 2010). The dose received by the heaviest animal in the range is designated as the minimum therapeutic dose. The dose received by the lightest animal in the range is designated the maximum exposure dose. The maximum exposure dose must then be multiplied by 1, 3, and 5 times. The regulatory guidelines also determine the number of times a formulation must be administered during the study and in addition to the minimum age of animals to be tested. The formulation is recommended to be administered monthly for six treatments. If a product is designed for use in young animals, the age of the animal tested must be the minimum age for which the commercial product will be used. Establishment of safety for use in the target species and for animal at a minimum age is mandatory to get a registration as veterinary medicine. It is necessary to demonstrate to the veterinarians and the pet owners that no unexpected adverse event will occur in treated dogs.

Therefore, the objective of this study was to determine the safety profile of afoxolaner administered in a soft chewable formulation to 8-week-old dogs at either 1 ×, 3 × or 5 × the maximum exposure dose (i.e., 6.3 mg/kg, 18.9 mg/kg and 31.5 mg/kg) at three, one-month-dose-intervals followed by three, 2-week-dose intervals.

2. Materials and methods

This study design followed the recommendations of VICH Guideline GL 43 “Target Animal Safety – Pharmaceuticals: Target Animal Safety for Veterinary Pharmaceutical Products” (VICH, 2008), and was conducted in accordance with the Food and Drug Administration; Good Laboratory Practice Regulations for Non-Clinical Laboratory Studies, 21 CFR Part 58, which is also accepted under the Organization for Economic Co-operation Development (OECD) Principles of Good Laboratory Practice (Revised 1997, issued January 1998) ENV/MC/CHEM(98)17, OECD Commission Directive 1999/11/EC of 08 March 1999. All animal

procedures in this study were reviewed and approved by the Merial Institutional Animal Care and Use Committee (IACUC). Dogs were managed consistent with the US Animal Welfare Regulations (USDA, 2008).

3. Animals

Thirty-two beagle dogs (16 males and 16 females) were included in the study. The dogs were 8.1–9.3 weeks of age on Day 0 and weighed 2.25–4.00 kg on Day –2. Dogs had not been exposed to ectoparasiticides prior to treatment and were in good health.

They were fed a commercial dry dog food ration calculated to maintain a healthy physical state and water was available *ad libitum*. Housing was in an environmentally controlled building. After allocation and until Day 20, two dogs of the same treatment group were co-housed. On Day 0 post dosing and from Day 21 to study end, dogs were housed individually and provided socialization time daily.

4. Experimental design

A random block design was chosen for allocation. Dogs were weighed on Day –2 and were ranked by decreasing body weight within sex and blocks of four dogs each were formed. Within each block, dogs were allocated randomly to one of the four treatment groups (Table 1) using the procedure Plan in SAS® Version 9.1.3. The final soft chewable formulation of afoxolaner for oral administration was manufactured under current Good Manufacturing Practices at Merial Limited. Three sizes were used: 0.5 g (11.3 mg afoxolaner); 1.25 g (28.3 mg afoxolaner); and 3 g soft chewables (68.0 mg afoxolaner).

Dose rate calculations were based on the body weight obtained at the most recent physical examination. As the chews cannot be divided, the number of chews administered, made up of identical or various sizes, was the number closest to, but not less than, the total mg dose needed (e.g., 1.95 kg bodyweight × 18.9 mg/kg = 36.86 mg afoxolaner; one 0.5 g and one 1.25 g chew = 39.7 mg afoxolaner for a treatment group 3 dog (Table 1).

Treatments were administered at one-month dose intervals at Days 0, 28 and 56, and then at 2-week dose intervals (at Days 84, 98 and 112). On each designated treatment day, control dogs were handled similarly to the treated dogs but were not dosed. Dogs in 1 × group were treated with the required dose of afoxolaner chews provided in a single fraction. Due to the volume of chewable material to be provided, dogs in 3 × and 5 × groups received approximately half of their required dose (first fraction) initially, with the rest of the required dose (second fraction)

administered approximately 3 h later. The dose was divided into two fractions for puppies in the 3× and 5× groups so that over-distension of the stomach would not occur. To avoid bias, control and dogs in the 1× groups were handled similarly to the other two groups but were not dosed during the administration of the second fraction. Food was offered to the dogs prior to and after each treatment.

Dogs were observed at least hourly for 3 h after the first dose fraction was administered and hourly for 4 h after the second dose fraction was administered. Feces from all dogs were examined for the presence of whole undigested chews on the day after treatment.

Personnel involved with recording of the in-life observations were blinded as to treatment. The pathologist performing the necropsy was unaware of dog's group but the origin of the dogs was unblinded for the histologic evaluation.

5. Study outcome evaluation

Physical examinations were performed weekly during the pre-test period and biweekly to Day 125, and included the evaluation of the general appearance, body weight, respiration rate, heart rate, and body temperature. Daily feed intake was monitored and recorded for analysis beginning on Day -1. In addition, blood hematology, plasma chemistry, and coagulation profiles were determined twice during pre-test in conjunction with physical examinations on Days 14, 27, 42, 55, 70, 83, 97, 111 and 125. Standard laboratory techniques were used for collection and analysis of the samples. The Merial laboratory conducting the analysis provided reference ranges for the plasma chemistry, coagulation, and hematology profiles. The hematology profile included: red blood cell count (RBC), white blood cell count, white blood cell differentials (absolute count), platelet count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean cell hemoglobin concentration, and RBC morphology. The plasma chemistry profile included: alanine aminotransferase, albumin, alkaline phosphatase, amylase, aspartate aminotransferase, calcium, chloride, cholesterol, creatinine, globulin, glucose, phosphorus, potassium, sodium, total bilirubin, total protein, triglycerides, and urea nitrogen. The coagulation profile included activated partial thromboplastin time, prothrombin time, and thrombin clotting time. Urine samples were obtained once during pretest and on Days 27 and 126 using either a metabolism pan or by cystocentesis (Day 126). Urinalysis included determination of urobilinogen, nitrite, glucose, bilirubin, ketones, blood, leukocytes, specific gravity, pH, and protein by use of MULTISTIX® SG Reagent Strips (Bayer Corporation). In addition to the reagent strips, a refractometer was used to determine urine specific gravity. Urine sediment was evaluated microscopically for at least the following: crystals, casts, red blood cells, white blood cells, and epithelial cells. Standard laboratory techniques were used. Reference ranges for the specific gravity was from [Stockham and Scott \(2002\)](#).

Dogs were humanely euthanized on Day 126 and a complete post-mortem examination was conducted. Samples of the pertinent tissues and representative samples

of tissues containing gross lesions were collected and processed for histological examination.

Blood samples for analysis of afoxolaner concentration were collected as either part of the samples collected for clinical chemistry analysis or were collected separately 3 h after treatment on Day 112. Plasma samples were analyzed quantitatively to determine afoxolaner concentrations using a method based on 96-well solid phase extraction of afoxolaner from canine plasma and a proprietary internal standard followed by LC-MS analysis as described in [Letendre et al. \(2014\)](#).

6. Data analysis

The physical exam, continuous clinical pathology values, and urinalysis were analyzed over the full study. The analysis of these variables used repeated measures analysis of covariance (RMANCOVA), including treatment, sampling day, sex, and their interaction terms as fixed effects. The covariate was the most recent pre-treatment value. If the three-way interaction, "treatment by sex by sampling day", was significant at the $p=0.05$ level, then no further evaluation was done. If the "treatment by sex by sampling day" interaction was not significant, then "treatment by sex" and "treatment by sampling day" were evaluated. If either two-way interaction was significant, then the treatment means were compared to the control group within each level of the corresponding factor. If neither was significant, the effect of treatment was evaluated and if significant, the treatment means were compared to the control group. Other than the test of the three-way interaction, all statistical analyses used $p=0.10$ significance level. When compared to efficacy testing of molecules where a significance level <0.05 is needed, the choice of 0.10 significance level for animal safety study increases the safety margin by highlighting effects that would not appear at $p < 0.05$.

During the study, commercial food was offered at least twice daily and total daily consumption was analyzed.

Organ weights (absolute, per 100 g body weight and per 100 g brain weight) were analyzed using analysis of variance (ANOVA).

Abnormal health findings were summarized by treatment group using Veterinary Medicinal Dictionary for Drug Regulatory Authorities (VEDDRA) terms ([EMA, 2013](#)). For the analysis of health abnormalities, the analysis endpoint was the number of dogs within each treatment group that experienced that abnormality at least once during the study. If a treatment group other than control had at least 4 animals experiencing the abnormality, then the three treated groups were compared to the control group using the Pearson Chi-Square test on a pair-wise basis. The only abnormalities analyzed in this manner were emesis and diarrhea.

Nine plasma samples over 126 days from each treated dog were collected in order to establish afoxolaner plasma concentrations during the study. Samples were taken prior to each treatment to establish that steady state had been reached during monthly dosing. Samples were also taken 3 h after dosing which is in the range of the maximum

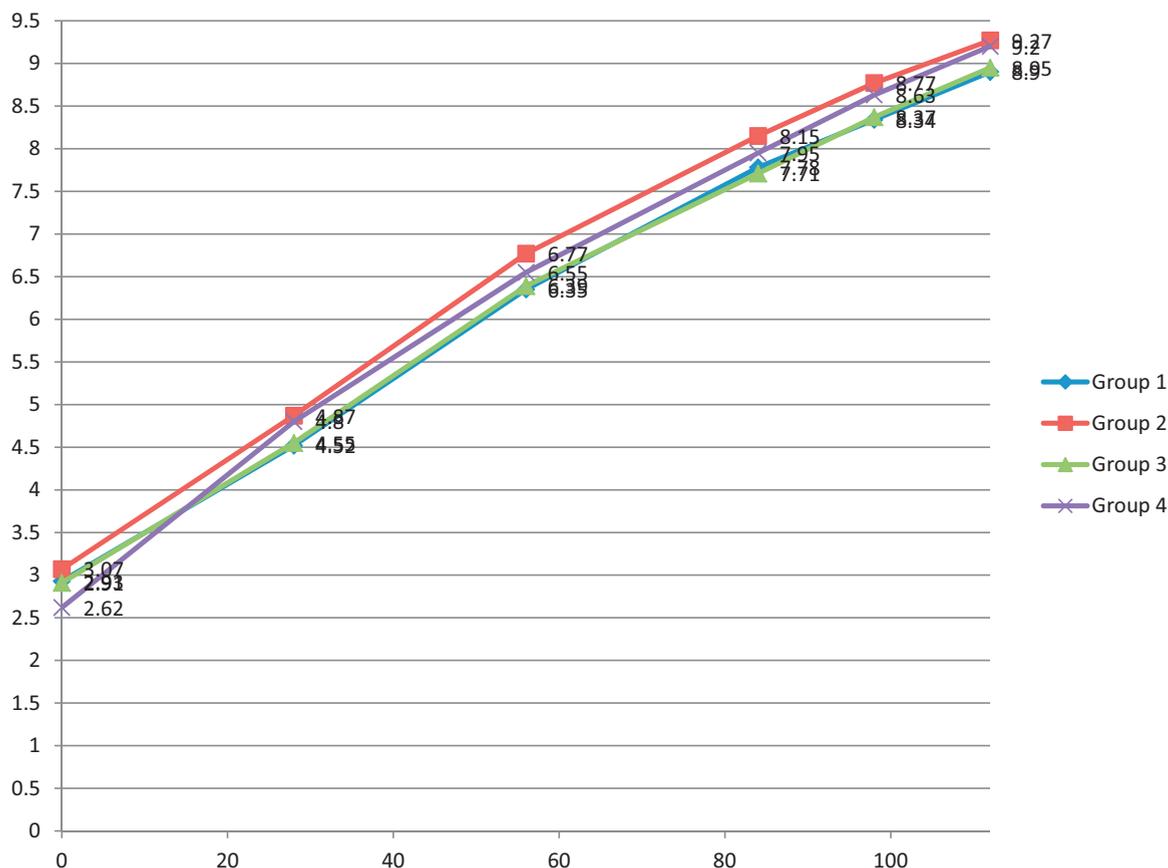


Fig. 1. Arithmetic mean body weights (kg) per treatment group. Group 1 = untreated control, Group 2 = 1 × afoxolaner (≥ 6.3 mg/kg; oral), Group 3 = 3 × afoxolaner (≥ 18.9 mg/kg; oral), Group 4 = 5 × afoxolaner (≥ 31.5 mg/kg; oral).

concentrations (between 2 and 6 h) following the final treatment (Letendre et al., 2014).

7. Results

No clinically relevant afoxolaner-related changes were observed in feed consumption, body weight, or physical examination parameters (i.e., heart rate, respiratory rate, and body temperature) at any of the sampling periods scheduled throughout the 126 days of the study covering the 6 treatment administrations. The only statistically significant difference was in the overall mean respiratory rate that was slightly higher than the overall mean control value in female dogs administered either 1 × or 3 ×, and in male dogs administered 5 × the maximum dose of afoxolaner. These changes in mean respiratory rate were slight, not changed in a time or dose-related manner, and within the expected respiratory range for maturing, active growing puppies. No clinically or statistically significant health abnormalities related to the administration of afoxolaner were observed, however, vomiting and diarrhea were observed sporadically across all groups, including the controls. One dog in the 5 × group vomited 4 h after treatment. The monthly/biweekly exposure to afoxolaner did not cause incremental increase in the incidences of vomiting and diarrhea in any of the treated

groups. Occasional vomiting and diarrhea did not interfere with daily food consumption or normal growth in the puppies (Fig. 1). No afoxolaner-related changes were observed at necropsy or in H&E stained microscopic tissue sections. There were no changes in organ weights. Accordingly, there were no clinically relevant afoxolaner-related changes in hematology, plasma chemistry, coagulation profiles, or urinalysis parameters at any of the sampling periods scheduled throughout the in-life period. Statistically significant ($p < 0.05$) changes in mean corpuscular hemoglobin concentration, red blood cell counts, basophils, albumin, calcium, phosphorus, and sodium were observed in different groups during the study. The fluctuations in clinical pathology variables were slight and did not change in a time- or dose-responsive manner. All group means fell within the laboratory's historical control ranges for maturing Beagle dogs.

Afoxolaner steady state plasma concentrations were reached by Day 27 as demonstrated by the lack of statistically significant differences ($p > 0.05$) between pre-dose samples taken on Days 27, 55 and 83 (Table 3). Dose proportionality was demonstrated and mean C_{\min} values on Days 27, 55 and 83 ranged from 138.8 to 197.6 ng/mL, 273.2 to 472.5 ng/mL, and 629.5 to 954.1 ng/mL following the 6.3 mg/kg, 18.9 mg/kg and 31.5 mg/kg treatments, respectively (Table 2). Concentrations increased as expected

Table 2

Afoxolaner plasma concentrations (ng/mL) taken one day prior to next treatment.

Treatment group (dose)	Pre-dose afoxolaner concentrations (ng/mL)		
	Day 27	Day 55	Day 83
1× (≥6.3 mg/kg)	167.0	138.8	197.6
p Value ^b	0.50	0.002^a	0.33
3× (≥18.9 mg/kg)	273.2	387.5	472.5
p Value ^b	0.22	0.86	0.32
5× (≥31.5 mg/kg)	629.5	674.4	954.1
p Value ^b	0.818	0.019^a	0.121

^a Bold indicates a *p* value < 0.05 indicating a statistical difference between Day 55 and Day 83 afoxolaner plasma concentrations was noted for the lowest and highest dose group; however, no trend was observed either with dose or with the three time points examined. This difference therefore does not indicate that steady state has not been reached.

^b *p*-Values are from a 2-tailed paired Student's *T*-test for concentrations on Day 27 and 55 (D27–55), Days 55 and 83 (D55–83) and Days 27 and 83 (D27–83).

Table 3

Mean ± SD of *C*_{max} of afoxolaner plasma concentrations (ng/mL) on Day 112 (~3 h post treatment).

Treatment group (dose)	<i>C</i> _{max} (ng/mL)
Group 2 – 1× (≥6.3 mg/kg)	4010 ± 660
Group 3 – 3× (≥18.9 mg/kg)	9370 ± 3360
Group 4 – 5× (≥31.5 mg/kg)	16400 ± 4400

following the transition to 2-week dosing intervals. Maximum measured plasma concentrations (*C*_{max} values) following the final treatment on Day 112 (~3 h post treatment) were 4010 ± 660 ng/mL, 9370 ± 3360 ng/mL, and 16400 ± 4400 ng/mL for the 6.3 mg/kg, 18.9 mg/kg and 31.5 mg/kg dose levels, respectively (Table 4).

8. Discussion

Afoxolaner was well tolerated when administered at 1×, 3× or 5× the maximum exposure dose to Beagle dogs as young as 8 weeks of age for six treatments. No clinically relevant treatment-related changes were observed for physical examination variables, clinical pathology, gross pathology, histopathology, or organ weights.

To get their approval, new animal health products are required to be tested for safety at 1, 3, and 5 times the maximum exposure dose using the formulation designated for commercial use. The minimum therapeutic dose of afoxolaner is 2.5 mg/kg (Letendre et al., 2014). The calculation of the maximum exposure dose is dependent on the weight ranges developed for the commercial presentations. For

products that are dosed on a specified mg/kg dosage (i.e., injectables), the therapeutic dose and the maximum exposure can be similar. For afoxolaner, the maximum exposure dose (6.3 mg/kg) is the highest dose that the lightest animal in a particular weight range will receive when dosed according to the label directions (Table 4).

Not only must the maximum exposure dose be administered but the number of treatments received by each animal is also defined by the product indications. For veterinary products intended to be used monthly, the regulatory agencies could require that the product be administered for six treatments, monthly or every 2 weeks for three months.

In this study, afoxolaner was administered 6 times; the first three doses at a monthly interval and the last three doses at a 14 day interval. This schedule was proposed in relation to the pharmacokinetic data of afoxolaner, in order to be sure that short intervals would not lead to accumulation of afoxolaner (Shoop et al., 2014; Letendre et al., 2014). Plasma afoxolaner concentrations reached steady state following the second monthly dose. This result was expected because the terminal plasma half-life is on average 18 days. At all dose levels tested, the kinetic profile of afoxolaner in the target animal safety study was predictable and consistent with the extensive preclinical evaluation (Letendre et al., 2014).

The higher plasma values were not associated with any adverse findings in the dogs thus adding to the margin of safety.

Regulatory requirements also guide which age of animals are to be tested. If a product is to be used in young animals, the study should include animals of the minimum age. Testing afoxolaner in young dogs at the maximum exposure dose for 6 treatments in an accelerated manner should highlight any potential safety concerns. The youngest dogs when this study began were 8.1 weeks. The maximum exposure dose and the accelerated treatment administration were all well tolerated in these puppies when first treated and continued as they matured.

Vomiting and diarrhea did occur sporadically during the study, however, the incidence was similar across all groups, including the untreated controls. Occasional vomiting and diarrhea are expected background observations in young dogs. The vomiting observed was usually of a small amount, limited to a single episode during a day, not associated with the time of food consumption, and resolved without any medical or dietary intervention. Observations of feces were performed at least twice daily and each observation spanned the time since the last observation. The change in consistency of the feces was normally observed in only one of several bowel movements that were present in the cage. In the majority of

Table 4

Ranges of afoxolaner doses administered to the dogs.

Dog group	Dose range (mg/kg)	Average multiple of therapeutic dose ^a	Average multiple of maximum exposure dose ^b
Group 2 (1×)	6.36–8.22	2.8	1.1
Group 3 (3×)	18.98–22.42	8.0	3.2
Group 4 (5×)	31.57–35.49	13	5.2

^a Therapeutic dose = 2.5 mg/kg.

^b Maximum exposure dose = 6.3 mg/kg.

cases, the other bowel movements that were present were normal. As with vomiting, the diarrhea observed across all groups and was usually of a small amount, limited to a single episode during a day, and resolved without any medical or dietary intervention. Occasional vomiting and diarrhea did not interfere with daily food consumption or normal growth in the puppies (Fig. 1). No particular pattern was identified to link vomiting or diarrhea to a disease as all dogs appear clinically healthy during the study and all clinical pathology results and histopathology of the digestive tract appeared normal.

The metabolic systems of juvenile dogs at 8 weeks of age are still developing thus administration of veterinary medicine to this age animal may have more profound effects than when administered to adults. Changes in body weight and daily feed consumption are excellent indicators that effects are occurring. Food in the study was offered twice daily and monitored, thus a decrease in food consumption would have been readily apparent. Body weight is a reflection of the food consumption and normal growth. The body weight curves of all the dogs in this study were consistent throughout all four groups (Fig. 1). The final body weights in all groups in this study are reflective of the excellent health of the dogs at the conclusion of the study.

9. Conclusion

The results of this study demonstrate that afoxolaner is safe when administered to dogs between 8 and 24 weeks of age, six separate times in a soft chewable formulation at up to 5× the maximum exposure dose.

Conflict of interest

The work reported herein was funded by Merial Limited, GA, USA. All authors are current employees of Merial.

Acknowledgments

The authors gratefully acknowledge the staff at Merial Limited for their help in conducting the studies to a high professional standard.

The authors gratefully acknowledge Lenaig Halos and Frederic Beugnet, Veterinary Parasitologists, for the scientific editing of the manuscript and to Amanda Mullins, Martha Massat, Robert Bastian, Norba Targa, Tim Underwood, and Tim Dotson for their contributions to this paper.

References

- Beugnet, F., Franc, M., 2012. Insecticide and acaricide molecules and/or combinations to prevent pet infestation by ectoparasites. *Trends Parasitol.* 28, 267–279.
- Blagburn, B.L., Young, D.R., Moran, C., Meyer, J.A., Leigh-Heffron, A., Paarlberg, T., Zimmermann, A.G., Mowrey, D., Wiseman, S., Snyder, D.E., 2010. Effects of orally administered spinosad (Comfortis) in dogs on adult and immature stages of the cat flea (*Ctenocephalides felis*). *Vet. Parasitol.* 168, 312–317.
- EMA, 2013. Veterinary Medicines and Product Data Management, Guidance Notes on the Use of VeDDRA Terminology for Reporting Suspected Adverse Reactions in Animals and Humans. EMA/CVMP/PhVWP/288284/2007-Rev.6, 13 June 2013, 11 pp. www.ema.europa.eu
- Letendre, L., Huang, R., Kvaternick, V., Harriman, J., Drag, M., Soll, M., 2014. The intravenous and oral pharmacokinetics of afoxolaner used as a monthly chewable antiparasitic for dogs. *Vet. Parasitol.* 201, 190–197.
- Shoop, W., Hartline, E., Gould, B., Waddell, M., McDowell, R., Kinney, J., Lahm, G., Long, J., Xu, M., Wagerle, T., Jones, G., Dietrich, R., Cordova, D., Schroeder, M., Rhoades, D., Benner, E., Con-falone, P., 2014. Discovery and Mode of Action of Afoxolaner, a New Isoxazoline Parasiticide for Dogs. *Vet. Parasitol.* 201, 179–189.
- Stockham, S.L., Scott, M.A., 2002. *Fundamentals of Veterinary Clinical Pathology*. Iowa State Press, 299 pp.
- USDA, <http://awic.nal.usda.gov/government-and-professional-resources/federal-laws/animal-welfare-act>
- VICH, <http://www.vichsec.org/en/guidelines2.htm>