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# Analysis of an eligible protocol for bioequivalence testing of two Canine anthelmintics products with two active molecules

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RESEARCH ARTICLE

## Abstract

This study is intended to analyze the design implemented in the bioequivalence testing of a generic product based on praziquantel and milbemycin oxime. The main objective of the study is to develop a protocol suitable for testing the bioequivalence and bioavailability of a new generic anthelmintic product intended for the therapeutic interchange of the innovative product Milbemax, whose patent has expired. The achievement of the proposed objective was ensured by the implementation of a single-center, cross-over, randomized, two-phase design separated by a break of 30 days between them. The current study was conducted on 22 clinically healthy Siberian Husky dogs. The analysis of the values obtained during the monitoring of the clinical and hematologic parameters, revealed evolutions within the allowed ranges, with the deviations being rare and devoid of clinical or statistical significance. Providing information regarding the field of bioequivalence of two similar antiparasitic products, being aware of the regulations in force as well as their recommendation by the treating veterinarian, in prophylaxis and anthelmintic therapy, is a good strategy to promote the use and acceptance of the new generic drugs.

**Keywords:** bioequivalence, Siberian Husky, study design.

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## INTRODUCTION

In recent years, the use of generic drugs has increased considerably, leading to the improvement of the veterinary medical system through the use of economic means of reducing costs. Generic pharmaceutical products are those drugs whose patent has expired and are made by other manufacturers, not by those who put the original product on the market (Dunne et al., 2013). They contain identical active substances and identical or different excipients for the innovative products. Innovative products are those drugs whose protection period can be between 10 and 20 years, sometimes this period can last up to 20 years (Dragus, 2014). Achieving standards of safety, efficacy, and quality of medical products is the responsibility of the Central Drugs Standard Control Organization (CDSCO) (Nagadurga, 2019). Bioequivalence and bioavailability testing studies are essential for determining the pharmaceutical equivalence between a generic and a reference product and represent one of the most important steps in the testing and commercialization of generic veterinary and/or human medicinal products

(Farczadi et al., 2019). By means of bioequivalence studies, the pharmacodynamics and pharmacokinetics of active metabolites or different active substances are compared (Ognean et al., 2010), and pharmacokinetic investigations are used to test them (Arion et al., 2015). Individual bioequivalence is another goal of this category of studies, and to achieve it, the cross-over study design is most appropriate (Chow and Liu, 2008), as is our present case. According to some market studies, by applying the compensation-free policy, the price of generic drugs for human use can be reduced by 20-90% compared to original equivalents (Dunne et al., 2013). The lack of such an opportunity in the case of veterinary drugs makes possible a reduction of these generic products by only 10-20%. Even if generic drugs have identical therapeutic effects to the original ones and, due to their lower cost, are more accessible on the market (Tolomeiu et al., 2018), some components used in their production can contribute to changing the pharmacokinetics of the drug. As for the costs of original drugs, their high price is directly proportional to the extent of both preclinical (in vivo and in vitro) and clinical (phase I, II, III, and even IV) pharmaceutical studies, as well as to their content in new active substances (Vlase et al., 2011). When introducing a similar competitive product to the market, it is not necessary to carry out the entire test protocol that the innovative product has already gone through. Thus, after determining the therapeutic equivalence, the study must be carried out according to the protocol, and it must necessarily be similar or equivalent to that of the innovative product (Nagadurga, 2019). The cost of the investigations required to obtain generic products is extremely low compared to those intended for the production of original drugs, as the only studies required are the ones for the assessment of bioequivalence and for pharmaceutical development and formulation. We appreciate that the lower price of generics is not due to a lower quality of the product but to the reduction of additional costs represented by clinical and paraclinical studies essential to the development of medicines (Tolomeiu et al., 2018). For example, the low prices of generic antibiotics relative to their effectiveness make them an important factor in antibiotic use strategies. Thus, studies have shown that the arrival on the market of generic veterinary antibiotics associated with reduced prices for farmers was associated with an increase in the use of these classes (Raboisson et al., 2020).

At the moment, in all specific legislation related to drug bioequivalence studies, the following statement is accepted and used: "Two drugs with the same plasma concentrations show the same therapeutic effect. Thus, two pharmaceutical products that present bioequivalence also present therapeutic equivalence to the same extent (Parii et al., 2016). Bioequivalence studies also serve to monitor the intensity and duration of side effects. The lack of side effects as well as interactions with other medical or food products indicates good bioavailability – characteristic of any drug with increased therapeutic efficacy (Lainesse et al., 2012). The rate and time in which the active substance of a drug is absorbed, and at the same time, is available at the site of action are key elements that make it possible to define bioavailability (Chow and Liu, 2008). Bioavailability analysis studies the influence and effect of some active substances in the body, and uses pharmacodynamic investigations to evaluate the mechanism, the effects of the medicinal substance, and the area of action. The difference in bioavailability between a test product and a reference product must fall within the reference average, which is in the range of 80–125%. The main purpose of the current study is to analyze an eligible protocol for testing the bioequivalence of two canine anthelmintic products with two active molecules based on milbemycin oxime 12.5mg and praziquantel 125 mg/tablet, which presents a major impact in veterinary medical therapy (Lainesse et al., 2012). The two molecules, present both in the generic product and in the reference product, are intended for the prophylaxis and treatment of parasitosis affecting canine species.

The recommended dosage for a dog weighing between 5-25kg is represented by one tablet.

The general objectives of our study are:

- Correlating the national and European legislative framework regarding the testing of the veterinary medicinal product on the canine species;
- Optimizing the collection and processing procedures of serial blood samples in order to test the bioequivalence of anthelmintic products and to observe the welfare norms of the subjects at the time of testing;
- Correlating clinical, hemato-biochemical, pharmacokinetic, and statistical indices with relevance in testing the bioequivalence of canine anthelmintic products;
- Evaluating the possibility of substituting of the product test, MILBEMAX® A.U.V. chewable tablets for dogs containing milbemycin oxime 12.5 mg and oral praziquantel 125.0 mg, with the new formulated generic product, MILBENIN® 12.5 mg/125 mg chewable tablets for dogs A.U.V., in the anthelmintic therapy for dogs;
- Evaluating the adverse effects as well as the tolerance of the two anthelmintic products, based on milbemycin oxime and praziquantel, subject to bioequivalence testing on canines.
- The objectives assigned exclusively to the partner, USAMV Cluj-Napoca, are represented by:
- Organizing the study, by implementing the physio-pharmacological protocol and participating in all stages of the bioequivalence test;
- Selecting and performing the clinical evaluation of animals during the initial and final screening;
- Collection of serial blood samples at well-defined intervals;
- Obtaining plasma for measuring the plasma concentration of active substances;

- Interpreting the data obtained during clinical evaluations;
- Editing the file according to the requirements of the test protocol.

## MATERIALS AND METHODS

Studies and protocols for carrying out bioequivalence tests are also taking place on the territory of our country. One of the largest companies that has been active in this way since 1994 is represented by Vim Spectrum SRL, which has as its main activity the development and production of generic drugs. The used products were represented by those subjected to testing: MILBENIN® chewable tablets for dogs (milbemycin oxime 12.5 mg, praziquantel 125.0 mg), Vim Spectrum SRL, and the reference ones: MILBEMAX® A.U.V. chewable tablets for dogs (milbemycin oxime 12.5 mg, praziquantel 125.0 mg), Elanco France S.A.S. The design of the present study is a cross-over one, being specific for bioequivalence testing, so that it is carried out in two different periods (phase I and phase II), with two cross-over sequences carried out in a randomized single-dose fashion, with the active substances having a comparative bioavailability. It was not needed that the number one formulation in each representative sequence be greater than or even equal to the one of the compared products. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, nr. 262 from 16.06.2021.

Two batches of Siberian Husky dogs were used, organized on the basics of a unicentric, randomized, crossover protocol with two sequences and two treatments. This protocol was adapted to evaluate the bioequivalence of anthelmintic products with two types of active molecules on canids as the target species, following the steps described in Table 1.

**Tabel 1.** General outline of the study

| **Company** | **Pharma VIM Kft.** |
|---|---|
| **Products**<br>▪ **TEST**<br>▪ **REFERENCE** | MILBEMAX rágótabletta kutyáknak A.U.V.<br>MILBENIN chewable tablets for dogs A.U.V |
| **Active ingredients** | Milbemycin oxime 12.5 mg/tablet<br>Praziquantel 125.0 mg/tablet |
| **Study design** | Two periods, two sequences, crossover, randomisation, single dose, comparative bioavailability, healthy dog study |
| **Planned sample size** | 22 dogs planned |
| **Planned number of enrolled subjects** | 24 dogs registered |
| **Main selection criteria** | Dogs of the Siberian Husky breed, male and female, with a body weight between 17 and 25 kg |
| **Dosage administered/phase** | 1 tablet/dog/ Test or Reference product |
| **Route of administration** | Oral |
| **Duration of treatment** | Two doses, with a break of 30 days between them |
| **Primary parameters** | AUC0-t and Cmax for Milbemycin Oxime and Praziquantel |
| **Secondary parameters** | $T_{max}$, $AUC_{0\text{-}inf}$ |
| **Additional parameters** | % AUC extrapolated, T1/2, MRT |
| **Safety parameters** | Adverse events, clinical and laboratory screening, surveillance examinations |
| **Study procedure** | Each subject will be randomly administered a single oral dose (1 tablet/dog) of the TEST or REFERENCE product, in two phases, separated by a break of at least 14 days. Blood samples will be taken per-dose (0.0) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 36, 48, 72, 96, 120, 144, 168, 216, 264, 336, 408 and/or 480 hours post-administration. |
| **Analysis of samples** | Validated HPLC-MS method |
| **Statistical analysis** | ANOVA system on model: sequences, animal, period, treatment, logarithmic transformation of pharmacokinetic data and determination of 90% CI on Test/Reference ratios for primary parameters. Equivalent non-parametric methods for Tmax. |
| **Acceptance range** | AUC0-t, Cmax: 70-143%, logarithmically transformed data |

One of the most important aspects of the logistics of the protocol was realized following the determination of the plasma concentration time of the drug and its elimination stages. Following oral administration of praziquantel, it reaches a maximum value (Tmax) in approximately 0.5–4 hours, with a half-life (T1/2) of approximately 1.5 hours. The first passage of the molecule is carried out at the hepatic level, and it will undergo a rapid and complete biotransformation by the monohydroxylated derivatives, which are mainly glucuronide or conjugated sulfate. It binds to the plasma at a percentage of 80%. Excretion is fast and complete, so that 90% of the product is eliminated in the first 2 days through the kidneys.

For the milbemycin oxime molecule, Tmax is approximately 2-4 hours and T1/2 is approximately 1-4 days. Bioavailability is 80% (Milbemax Chewable Tablets for Dogs, n.d.). The values of the maximum concentration and half-life times are the essential factors that contributed to the realization of our protocol and the establishment of the hourly intervals for the collection of serial blood samples at well-defined intervals.

The location of the research, the necessary animals, the utensils, and the equipment were provided by the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, the Society Vim Spectrum Tg-Mureş, a kennel of Siberian husky, and accredited laboratories of the beneficiary, and a profile company from Hungary, certified in the EU. At the end of the accreditation, the file will be submitted to the Medicines Commission of some European countries,interested in capitalizing on this new generic product.

The present study was designed according to the international guidelines for bioequivalence testing, with the administration of a single dose, fasted, in both phases. Thus, the two phases are separated by an elimination period of 30 days. A number of 22 clinically healthy dogs were enrolled, according to the inclusion and exclusion criteria mentioned in Table 2, and the study was completed with 20 dogs eligible for bioequivalence evaluation. The subjects were representatives of Siberian Husky dogs, aged between 2 and 5 years, weighing between 19 and 25 kg, males representing the highest percentage (n = 20) and females the lowest (n = 2).

The dogs underwent a preliminary medical examination 7 days prior to the start of the treatment to establish the inclusion criteria and the clinical stage of the subjects following the realization of their metabolic and hematological profiles. During the screening procedures, the investigator checked demographic characteristics (age, sex), obtained a medical history, and performed a routine medical examination. The medical history was based on the investigation of previous prophylactic methods such as internal and external deworming, polyvalent and antirabies vaccinations, the existence and/or absence of immunological, metabolic, hematological or systemic pathologies, and not on the last pregnancies, calving, or lactations (in the case of females) or the last surgical procedures to which the dogs were subjected.

Blood and urine samples were collected for routine hematological and biochemical analyses, urine examinations, and serological ones. It was then verified that they were within the physiological limits, and based on the medical examination, the investigator decided the eligibility of belonging to the study, following the inclusion and exclusion criteria for the study.

**Table 2.** Inclusion and exclusion criteria of subjects in the study

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| Male or female | Hypersensitivity to the tested active substance or to those from the same pharmacological classes |
| Age between 1 and 5 years | Collies or other related breeds |
| Clinically healthy (normal hematology, biochemistry and urinalysis; may show slight deviations outside physiological limits) | Dogs pozitives to *Dirofilaria imitis* |
| Body weight between 17-25 kg | Disorders of acute illness in the last 14 days |
| | Dogs undergoing treatments that may present major or systemic organ disorders in the last 30 days, such as barbiturates or phenothiazines |
| | The presence or history of medical disorders such as cancer, renal, hepatic, gastrointestinal, respiratory, endocrine or locomotor pathologies as well as systemic ones caused by neurological, metabolic or hematological disorders |
| | Major surgical interventions in the gastrointestinal tract |
| | Current disorders or conditions that could cause impaired absorption, distribution, metabolism or excretion of drugs. |
| | Positive results following serological testing |
| | Pregnancy or lactation |

Accommodation space was allocated to each individual animal according to welfare standards, so that each animal benefited from individual open-air boxes with extremely generous space. Following the creation of the batches, the welfare rules for the use of animals for scientific purposes were respected, which assume that the dogs housed in pairs or in groups are each restricted to half of the total available space (2 m2 for a dog under 20 kg, 4 m2 for a dog over 20 kg). The period in which a dog was kept in such a limited space did not exceed 4 consecutive hours. A lactating female with her cubs should benefit from the same standards of space as a single female of equivalent weight. The farrowing boxes must be designed so that the female can move to an additional box or a raised area away from the cubs, according to Table 3 (Parliament of Romania, 2014).

**Table 3**. Standards according to the welfare rules for sheltering dogs in order to carry out tests for scientific purposes (Parliament of Romania, 2014)

| Weight (kg) | Minimum area of the boxes ($m^2$) | Minimum floor area for one or two animals ($m^2$) | Minimum floor area for one or two animals | Minimum height (m) | The date provided for in art. 32 para. (6) of the law |
|---|---|---|---|---|---|
| **Up to 20kg** | 4 | 4 | 2 | 2 | 1.01.2017 |
| **Over to 20kg** | 8 | 8 | 4 | 2 | |

The microclimate was represented by cages that kept the dogs outdoors, with permanent sanitation and adequate hygiene measurements. The food administered in the whole study was represented by Monge brand pellets, administered once a day with water ad libitum.
The method of assigning the subjects, following the establishment of the treatment groups, is specified in Table 4.

**Table 4.** Data regarding the identification of the animals introduced into the test, the dosage of the administered products, and the evolution of body weight (chip number, age, weight, date of participation in the first and second phases, sex)

| Subjects No. | Chip No. | Age (years) | Weight (kg) | Phase 1 | Phase 2 | Gender |
|---|---|---|---|---|---|---|
| **1** | 804098100100935 | 4 | 22 | 01.03.21 | 27.04.21 | M |
| **2** | 981020009411356 | 5 | 24 | 01.03.21 | 27.04.21 | M |
| **3** | 900085000351098 | 4 | 22 | 01.03.21 | 27.04.21 | M |
| **4** | 642094100001004 | 5 | 25 | 01.03.21 | 27.04.21 | M |
| **5** | 642099000761516 | 3 | 20 | 01.03.21 | 27.04.21 | M |
| **6** | 642094100001007 | 5 | 19 | 01.03.21 | 27.04.21 | F |
| **7** | 642094100001002 | 5 | 22 | 01.03.21 | 27.04.21 | M |
| **8** | 642099000761513 | 2 | 23 | 01.03.21 | 27.04.21 | M |
| **9** | 900085000354428 | 4 | 21 | 01.03.21 | 27.04.21 | M |
| **10** | 642099000611974 | 3 | 25 | 01.03.21 | 27.04.21 | M |
| **11** | 642099000761518 | 2 | 25 | 01.03.21 | 27.04.21 | F |
| **12** | 642093400126443 | 3 | 23 | 01.03.21 | 27.04.21 | M |
| **13** | 642090003608397 | 3 | 20 | 01.03.21 | 27.04.21 | M |
| **14** | 642099000439105 | 2 | 22 | 01.03.21 | 27.04.21 | M |
| **15** | 981020009429443 | 5 | 25 | 01.03.21 | 27.04.21 | M |
| **16** | 642099000761517 | 3 | 21 | 01.03.21 | 27.04.21 | M |
| **17** | 642099000761505 | 2 | 20 | 01.03.21 | 27.04.21 | M |
| **18** | 642093400159196 | 2 | 21 | 01.03.21 | 27.04.21 | M |
| **19** | 642090003675302 | 2 | 21 | 01.03.21 | 27.04.21 | M |
| **20** | 642090003675309 | 2 | 22 | 01.03.21 | 27.04.21 | M |
| **21** | 642095600000471 | 5 | 22 | 01.03.21 | 27.04.21 | M |
| **22** | 642090000518728 | 4 | 24 | 01.03.21 | 27.04.21 | M |

Thus, each subject benefited from an identification number that was successively assigned by the clinical investigator, according to the order of enrolment in the study. Each subject was assigned to one of two treatment sequences (TR or RT). The randomization list for the study drug allocation was generated by the sponsor using the website www.randomization.com. The dosing regimen was the same for all subjects enrolled in the trial. Each subject received one tablet/dog of the reference formulation in the first part of the study or the test product in the second part, according to the randomized list. The clinical part of the study was carried out according to the requirements of the analysts, not specifying which of the products used is the test and which is the reference for the authenticity of the study.

The food restriction was carried out 12 hours before and after the administration of the test and reference products, and water was restricted only 2 hours before and after the administration of the treatment.

In both phases of the study, well-established intervals were determined by blood sampling following the establishment and evaluation of pharmacokinetics, before and after administration of the products at specific times up to 480 hours after their administration. At the end of phases, I and II, a total of 72 serial blood samples were collected from each dog. Bioequivalence assessment was performed based on the primary pharmacokinetic parameters of milbemycin oxime and praziquantel (Cmax and AUC0-t) after administration of the test product and the reference product.

Subjects were housed in the study center from the screening period until the final examination. The drug (1 tablet/dog of the test or reference products) was administered orally under the direct observation of the clinical investigator. At the time of swallowing the tablet, the investigator or his designee performed a check of the oral cavity to ensure that the dose was taken in its entirety. In each phase of the study, subjects received the treatment at the same time (± 30 minutes).

Blood samples were collected before dosing and up to 480 hours after dosing by venipuncture from the cephalic, brachial, saphenous, or jugular veins. In order to obtain a reliable plasma profile of milbemycin oxime and praziquantel, after oral administration of the test and reference products, it was necessary to collect 3 ml of venous blood at the times described in Table 5. The date and time of serial sample collection were recorded in CRF (case report form). Each blood sample was collected on anticoagulant (EDTA) and then centrifuged at 5000 rpm for 10–60 minutes. Following this, the plasma was separated and transferred into two tubes, tightly closed, which were then labeled, stored, and frozen at -20 °C until analysis.

**Table 5.** Sample collection times

| Day | Sample collection times |
|---|---|
| **1-20** | 0.0, 0.5; 1.0;1.5; 2.0; 3.0; 4.0; 6.0; 8.0; 10.0; 12.0; 14.0; 16.0; 18.0; 20.0; 2.0; 24.0; 28.0; 36.0; 48.0; 72.0;96.0; 120.0; 144.0; 168.0; 216.0; 264.0; 336.0; 408.0; 480 |
| **Sequences** | Subjects |
| **Ref. Test.** | 02, 05, 06, 07, 08, 10, 15, 16,18, 20 |
| **Test. Ref.** | 01,03, 04, 09, 11, 12, 13, 14, 17, 19, 21, 22 |

On drug administration days, all dogs received water in a standardized manner. Access to water was made at discretion, up to 2 hours before administration. Starting 2 hours after the administration of the drug, the subjects resumed their normal rate of water consumption. Subjects did not eat for 12 hours before drug administration, with this period extending up to 12 hours after administration. They received a dose of the test product or the reference product in each phase of the study. Medication was administered according to a randomized list. The time of administration as well as the type of medication were noted in the CRF.

The entire protocol of the study is detailed in Table 6. The design of the study is a unicentric type with two treatments and a 30-day break between them, and it took place during five distinct stages as follows:

- In the first stage, the two groups were organized for the formation of the sample based on the inclusion and exclusion criteria and the initial clinical and laboratory examinations that included routine hematological, biochemical, and serological profiles.
- The first phase of the study involved several activities, such as:
- On day 0, mainly organizational activities were carried out, including the review of the last details regarding the conduct of the study and the sheltering and accommodation of the subjects in the well-equipped and arranged spaces for testing.
- On day 1, the first blood sample was collected (time 0.00) before the administration of the products. Following compliance with the subjects' randomization list, the test product (Milbenin) and reference (Milbemax) were administered. The first 16 doses of post-dosing samples were collected at the following

time intervals: 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 hours.

- In the following 2–21 days, the blood samples were collected and clinical evaluations were carried out at the following 15-time intervals that took place at 24, 28, 36, 48, 72, 96, 120, 144, 168, 216, 264, 336, 408 and 480 hours.
- The washout period was 30 days and represented the time period between the two phases, during which the elimination of the first dose of the drug took place.
- The second phase of the study included the repetition of the activities carried out in the first phase (clinical examinations, the collection of serial blood samples, and obtaining plasma).
- The final examinations were carried out at the end of the second phase of testing. To conclude the study, each subject underwent a final medical examination within 14 days after the last phase two blood draw. At the final examination, the same clinical, hematological, and biochemical procedures were applied as for the examination of screening.

**Table 6.** General protocol of the study

| **Procedure** | **Screening** | **Phase** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phase I** | | | | | | | | | | | | | | | **Rest period (30 days)** |
| The day of the study | -7...-1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 11 | 14 | 17 | 20 | |
| Selection of batches for testing | ◉ | | | | | | | | | | | | | | |
| Verification of inclusion/exclusion criteria | ◉ | ◉ | | | | | | | | | | | | ◉ | |
| Clinical exam | ◉ | | | | | | | | | | | | | | |
| Vital signs | ◉ | | ◉ | | | | | | | | | | | | |
| Biochemistry | ◉ | | | | | | | | | | | | | | |
| Hematology | ◉ | | | | | | | | | | | | | | |
| Urine analyses | ◉ | | | | | | | | | | | | | | |
| Serological analyses | ◉ | | | | | | | | | | | | | | |
| Product administration | | | ◉ | | | | | | | | | | | | |
| Serial collection of blood samples | | | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | |
| Observation and recording of adverse effects | | | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ |
| **Phase II** | | | | | | | | | | | | | | | **Final exam** |
| The day of the study | | | | | | | | | | | | | | | |
| Selection of batches for testing | | | | | | | | | | | | | | | |
| Verification of inclusion/exclusion criteria | | | | | | | | | | | | | | | ◉ |
| Clinical exam | | | ◉ | | | | | | | | | | | | ◉ |
| Vital signs | | | | | | | | | | | | | | | ◉ |
| Biochemistry | | | | | | | | | | | | | | | ◉ |
| Hematology | | | | | | | | | | | | | | | ◉ |
| Urine analyses | | | | | | | | | | | | | | | ◉ |
| Serological analyses | | | | | | | | | | | | | | | |
| Product administration | | | ◉ | | | | | | | | | | | | |
| Serial collection of blood samples | | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | |
| Observation and recording of adverse effects | | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ | ◉ |

## RESULTS AND DISCUSSIONS

The United States Food and Drug Administration (FDA) and other EMEA (Europe, the Middle East, and Africa) agencies have considered the cross-over design to be most relevant in conducting bioequivalence studies due to the following aspects:

- There is no intersubjective variability when comparing the two formulas.
- Each subject serves its own control, right so a comparison of the effects of the two formulations on the same subject is made.
- Adequate randomization of subjects following sequential administration of the formulation provides the best estimates following rotation of administration of the two formulations (Chow and Liu, 2008).

The present study was based on the serial collection of blood samples to determine the concentration of the active molecules in the test and reference products in Siberian husky dogs. Although serial blood sampling allows us to determine important parameters for bioequivalence testing in a short period of time, it can also act as a stress factor for subjects (Ognean et al., 2012).

During the entire testing, as well as at the beginning and end of the study, serological, biochemical, hematological, parameters and vital signs were investigated, and adverse effects were recorded. Thus, the essential role of bioequivalence tests is given by the fact that they monitor the duration and intensity of side effects. A generic product showing increased efficacy must present extended bioavailability and be characterized by a lack of side effects or interactions with other drugs or food products (Tolomeiu et al, 2018).

The collection of appropriate samples is essential to reaching the threshold of success in terms of bioequivalence testing, thus taking into account the most important factors represented by professional ethics, the existence of good practices and laws, and respecting the welfare of the animals participating in the test (Ognean et al., 2012).

Thus, metabolic profile tests are necessary; they provide essential information for the analysis of the rate and extent of absorption of active molecules and the overall bioavailability of medicinal substances, which is necessary for bioequivalence studies. One of the most important requirements with a major impact on the success of bioequivalence studies is represented by the correct collection of serial blood samples that must comply with welfare, professional ethics, and animal protection norms (Tolomeiu et al., 2018).

The completion of the study was achieved by evaluating the essential parameters of the bioequivalence studies, which were represented by the Cmax and AUC0-t of milbemycin oxime and praziquantel in the test product and the reference product. These parameters were used to quantify the data following their logarithmic transformation using the ANOVA system and the KINETICA 4.4.1 program. Thus, it was necessary to take into account the following variables: administration period, subjects, sequences, and treatments, and the pharmacokinetic parameters used were represented by AUC0-t, and Cmax, Tmax, AUC0-inf, AUC, T1/2, and MRL-t.

The interpretation of the blood samples was carried out following the use of UV detection of the drug concentration in the blood plasma (after its separation from the blood by centrifugation and storage at -20 °C). Finally, the analysis of the samples for the Milbenin product was quantified using mass spectrophotometry through liquid chromatography at some specialized laboratories in the EU, and then a validation of these results was carried out through the manufacturer's laboratories (Vim Spectrum SRL).

## CONCLUSIONS

The current study is compliant with the ethical norms and rights related to the subjects used in the testing of Milbenin. The eligibility of the subjects for the study is marked by the fact that no deviations from the protocol design were observed, so no adverse effects were noted, and parameters excessively increased compared to the physiological limits in correlation with the clinical condition of the subjects were not observed. These arguments certify that the present study did not affect the clinical status of the subjects. Their suffering was minimized due to the professional and appropriate use of restraint methods and blood collection by qualified staff represented by veterinarians, technicians, and specialists in the field. Providing information regarding the field of bioequivalence of two similar antiparasitic products, being aware of the regulations in force as well as their recommendation by the treating veterinarian in prophylaxis and anthelmintic therapy, is a good strategy to promote the use and acceptance of the new generic drugs.

**Author Contributions:** D.D.T. Wrote the paper and performed analysis; M.C. Collected the data; O.T.K. Collected the data; D.F. Contributed data or analysis tools; D.N. Contributed data or analysis tools; L.O. Conceived and designed the analysis.

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**Conflicts of Interest**

The authors declare that they do not have any conflict of interest.

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