(PhD THESIS SUMMARY)

Bioequivalence and tolerance analysis of a new generic canine anthelmintic containing Milbemycin oxime and Praziquantel

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INTRODUCTION

Bioequivalence testing allows the introduction of generic products onto the market, which are similar to and competitive with original products but have the advantage of not undergoing the entire testing protocol, thus reducing their cost. Bioequivalence studies also ensure the comparison of the pharmacokinetics and pharmacodynamics of active molecules and their resulting metabolites. Currently, bioequivalence testing is performed exclusively on target animal species, ensuring proper evaluation of equivalence, tolerance, safety, and therapeutic efficacy (OGNEAN, 2019). Such a study is also addressed in this thesis, focusing on the bioequivalence testing of two products, Mibenin (generic) and Milbemax (reference), both containing Milbemycin Oxime and Praziquantel.

Milbemycin Oxime (MO) is chemically similar to Ivermectin and belongs to the class of Macrocyclic Lactones (MLs), the discovery of which was awarded the Nobel Prize in Physiology or Medicine in 2015 (WANG şi colab., 2020). MO is an active molecule that has led to significant advances in biological sciences, with notable medical implications, including the control of common parasitic infections in humans and animals. Besides antiparasitic effects, MLs also exhibit antifungal, pesticidal, antidiabetic, antiviral, and anticancer properties (EL-SABER BATIHA şi col., 2020). In many regions (USA, China, Japan, Europe), MO is used in the control of parasitism caused by mites, helminths, and insects in companion animals.

Praziquantel (PZ) is a derivative of acylated pyrazino-isoquinoline, highly effective in the treatment of cestode and trematode infections (WALTHER şi col., 2014). The combination of MO and PZ has been recently introduced on the market, especially in canine and feline therapy, for the prevention of Dirofilaria immitis infestations, representing a molecular synergy with remarkable clinical outcomes (TORANMAL şi col., 2019).

PURPOSE AND MAIN OBJECTIVES OF THE THESIS

The aim of this doctoral thesis is to update current knowledge regarding the bioavailability, bioequivalence, tolerance, and therapeutic efficacy of anthelmintic products containing Milbemycin Oxime and Praziquantel, culminating in the formulation and bioequivalence testing of a new generic product with MO and PZ, interchangeable in canine clinical practice.

The general objective of the research focused on the formulation and bioequivalence testing of the generic product Milbenin. The specific objectives included:

- Updating national and European legislation related to the testing of veterinary medicinal products in dogs as the target species, in correlation with current regulations on animal welfare and good practice;

- Developing a unicentric, crossover, randomized protocol with a double therapeutic sequence, suitable for bioequivalence testing of the new generic product Milbenin chewable tablets for dogs, containing MO and PZ;
- Analyzing the relevance of clinical and hematobiochemical indicators for assessing the eligibility of selected subjects and monitoring their health during the bioequivalence testing of canine anthelmintic products;
- Optimizing procedures for serial blood sampling in bioequivalence testing of pharmaceutical products in dogs as the target species;
- Analyzing plasma concentrations of active molecules in correlation with pharmacokinetic and statistical parameters, serving as the basis for bioavailability and bioequivalence testing of the investigated products;
- Analyzing prophylactic-therapeutic conditions and the potential for interchangeability of the reference product on the market with the tested generic product;
- Evaluating adverse effects and tolerance of the investigated products;
- Assessing the therapeutic efficacy of the newly tested product under field conditions

STRUCTURE OF THE THESIS

This doctoral thesis, entitled ***"Bioequivalence and tolerance analysis of a new generic canine anthelmintic containing Milbemycin Oxime and Praziquantel"*** is structured and written in accordance with the recommendations of the Doctoral School of USAMV Cluj-Napoca and has received a positive approval from the Bioethics Committee in the field, as per Decision No. 226/03.08.2020. The thesis comprises a total of 130 pages, of which 43 pages (30,93%) correspond to the first part, consisting of 3 chapters, and 96 pages (69,07%) to the second part, developed across 8 chapters.

PART I-CURRENT STATE OF KNOWLEDGE

The first part provides a concise and up-to-date bibliographic overview, offering a comparative analysis of the main advances in the fields addressed in this study, focusing primarily on legislation and authorization of veterinary medicinal products, the bioavailability and bioequivalence of anthelmintic products of major interest in canine clinical practice, and the therapeutic impact of MO and PZ.

Chapter 1, entitled **"Recent Developments in the Study and Authorization of Veterinary Medicinal Products"**, analyzes and updates, in three subchapters, the recent progress in the formulation, testing, and authorization of generic veterinary medicinal products compared to original products, detailing the assessment of their safety and therapeutic efficacy.

Chapter 2, "Specific Aspects of Veterinary Drug Bioequivalence", is organized into four subchapters that document and explain the sequence of steps involved in bioavailability and bioequivalence testing in animals, including the assessment of subject eligibility, as well as the clinical, paraclinical, pharmacokinetic, and statistical evaluations specific to these highly relevant studies.

Chapter 3, entitled **"Therapeutic Impact of Milbemycin Oxime and Praziquantel"**, details, in two subchapters, recent findings regarding the tolerance, safety, and therapeutic efficacy of products containing MO and PZ, as active molecules from the macrocyclic lactone (ML) class and pyrazino-isoquinoline derivatives, respectively, compared to other pharmacological groups with significant impact in anthelmintic therapy for carnivore species.

PART II- PERSONAL CONTRIBUTION

Part II, prepared in accordance with the standards and requirements of doctoral studies in veterinary medicine, includes the author's personal contributions, developed over six chapters, detailing five distinct studies, each published as original research articles in high-impact journals relevant to the addressed field.

The personal contribution section begins, as customary, with a detailed presentation of the research hypothesis, the aim and general objectives of the studies, as well as the locations and specific conditions under which the research was conducted to obtain the anticipated results.

Chapter 4 is dedicated to the presentation of the biological material and technical requirements, along with a detailed analysis of the methodology employed in the studies developed in the doctoral thesis. This chapter provides a comprehensive description of the methods and investigations used in carrying out the research underlying the five doctoral studies. These included the documentation and development of a unicentric physio-pharmaceutical protocol for drug bioequivalence testing in Siberian Husky (HS) dogs; the methodology for clinical, hematological, and blood biochemical evaluations of canine subjects during testing; the pharmacokinetic and statistical analyses specific to drug bioequivalence evaluation; and the design for assessing the therapeutic efficacy of the newly formulated generic product under field conditions.

Chapter 5 includes the first study, entitled "Analysis of the Design Implemented in the Bioequivalence Study." The study design is illustrated in the scheme presented in Table 1, which provides a detailed overview of the complexity of the protocol applied in the bioequivalence assessment of the investigated generic product. A randomized, unicentric, cross-over design was implemented, comprising two periods and two sequences, with a single-dose administration and two treatments, separated by a 30-day washout interval. The study protocol included the following stages:

- Days -7 and -1: evaluation and selection of subjects; validation of the LC-MS/MS method (liquid chromatography coupled with mass spectrometry) for quantifying the pharmacokinetic parameters of the active molecules MO and PZ;
- Day 0: conduct of Phase I—review of the groups and initiation of the study;
- Day 1: collection of the first blood sample (time 0.00) and administration of the test product and reference product, according to the randomization list, followed by serial post-dose collection of 16 blood samples;
- Days 2–21: continuation of serial blood sampling at the next 15 time intervals and clinical evaluations;
- Days 21–51: 30-day washout period for clearance after the first dose;
- Conduct of Phase II: repeating the activities from Phase I;
- Final examinations and determination of the level of bioequivalence.

Table 1. ***Study protocol scheme used for the bioequivalence assessment***

	Screening	Phase I												Washout-perd 30 days	
Day	-7-1	0	1	2	3	4	5	6	7	9	11	14	17	20	
Group selection	◉														
Assessment of inclusion criteria	◉	◉												◉	
Clinical examination	◉														
Vital signs	◉		◉												
Blood biochemistry	◉														
Hematology	◉														
Serology tests	◉														
Drug administration			◉												
Blood sampling			◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	
Adverse event reporting			◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉
		Phase II												Final exam	
Day	-7-1	0	1	2	3	4	5	6	7	9	11	14	17	20	
Group selection															
Assessment of inclusion criteria															◉
Clinical examination			◉												◉
Vital signs															◉
Blood biochemistry															◉
Hematology															◉
Serology tests															
Drug administration			◉												
Blood sampling		◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	
Adverse event reporting		◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉	◉

Chapter 6, dedicated to the next study, **"Clinical evaluation of dogs during testing"**, groups the set of usual clinical examinations performed initially for the selection of healthy animals, pre-dose and post-dose for monitoring health status, drug tolerance, and any adverse reactions. These examinations revealed the maintenance of a good general health condition in the canine subjects throughout the testing period, which proceeded without replacing any subject. Only a few minor deviations from physiological reference intervals were recorded during the final examinations. The post-dose evaluation of the active molecules in the sample of dogs subjected to the bioequivalence test showed that vital functions remained within physiological ranges, differing from the pre-dose evolution by a few exceptions without clinical significance or impact. Throughout the testing and for two months after its completion, no animal exhibited adverse reactions, clinical manifestations, or drug intolerance. The overall analysis of clinical results indicates that both products were well tolerated and demonstrated a good safety profile at the doses used, all of which enhance the value of the bioequivalence test.

Chapter 7 details the third study, titled **"Evolution of hematological indices in dogs during testing"** which first analyzes blood sample collection techniques in dogs, with particular adaptation for the HS breed, whose handling must be tailored to their morphophysiological and behavioral characteristics. The necessity of optimizing serial blood sampling procedures for testing the bioavailability and bioequivalence of active molecules in dogs as the target species, which have great breed variability, is also argued. The evaluation of collection techniques revealed the superiority of venipuncture using holder-type devices. Serial collections during the entire testing totaled 72 blood samples from one subject. The daily volume of blood collected reached a maximum (85 mL/animal) on the first day of both phases, then decreased as the testing progressed, resulting in a total of 310 mL blood per test per animal. Following the collection of this volume, no adverse reactions or significant negative impacts on blood volume, hematological profile, or animal health status were noted. It is considered that serial blood sampling affected blood volume less than the collection of 3 blood units (450 mL/unit), which is permitted to be collected annually from a canine donor weighing 20-25 kg (Ognean, 2017). Comparatively, the blood volume of 1.9 L in a canine subject weighing an average of 22 kg can decrease by a maximum of 16.2% following serial collections during the bioequivalence test. The risk of hypovolemia is therefore minor, and HS breed dogs are suitable for serial blood collections, a conclusion also supported by the insignificant deviations of hematological parameters observed during the testing.

Chapter 8, **"Evolution of the blood biochemical profile in tested dogs"** documents and presents the overall analyses of metabolic profile in the sample of subjects during the bioequivalence study. The results demonstrated the particular relevance of metabolic profile analyses in evaluating health status and detecting adverse

reactions in canine subjects during bioequivalence testing of medicinal products. Changes in metabolic profile parameters showed some slight deviations from physiological reference intervals which, being clinically insignificant and statistically non-significant, had minor pharmacokinetic and pharmacodynamic influences on the tested active substances. Among the recorded deviations, only slight increases in AST levels, correlatable with minor muscle trauma, and GGT levels, related to prior parasitic infestations, were noted. Fluctuations in blood biochemical parameters were also characterized by non-significant increases in total proteins, albumin, globulins, calcium, and phosphate levels, which were consequences of hemoconcentration, dehydration, muscular effort, or stress caused by serial blood sampling. It should also be noted that no sex-related differences were observed for metabolic profile parameters in the dog sample during testing, a finding also reported by other researchers in the field. Minor fluctuations without clinical-pharmacological impact of blood biochemical indices recorded at initial screening and final examination were correlated with the final clinical, hematological, pharmacokinetic, and statistical evaluations to confirm the bioequivalence of the investigated generic product, as well as the appropriateness of its therapeutic interchangeability.

Chapter 9, **"Analysis of bioequivalence and tolerance of the investigated product"**, groups the core results of the doctoral research, which confirmed the bioequivalence and therapeutic interchangeability of the tested products. The high plasma concentrations (CP) of the active molecules tested (Fig. 1 and 2) reveal prolonged contact durations with adult parasites and implicitly their good efficacy in the prevention and treatment of mixed helminthiasis, including dirofilariasis. In the case of MO, CP durations exceeded 400 hours, while for PZ they did not exceed 26 hours, demonstrating the slower absorption rate and longer persistence of the active MO molecule in the canine digestive tract. The analysis of t_{max} for the CP of MO A3 shows that the maximum value was reached 2 hours after dosing with the test product and 3 hours after administration of the reference product, with detectable CP values even after 480 hours for both products. The t_{max} analysis of CP for PZ revealed that the maximum concentration was reached 1 hour after dosing with the test formulation (T) and 1.5 hours after administration of the reference product (R). CP levels were detected up to 36 hours for product T and up to 26 hours for product R. The values of primary, secondary, and additional pharmacokinetic parameters, obtained through descriptive statistical analysis (Table 1), must be correlated with the 90% confidence interval for the T/R ratio of MO and PZ (Table 2). The key takeaway from analyzing this set of statistical parameters is that the 90% confidence interval of the primary pharmacokinetic parameters falls within the accepted limits (0.8–1.25), confirming the bioequivalence of the tested products in canine subjects and supporting their therapeutic interchangeability.

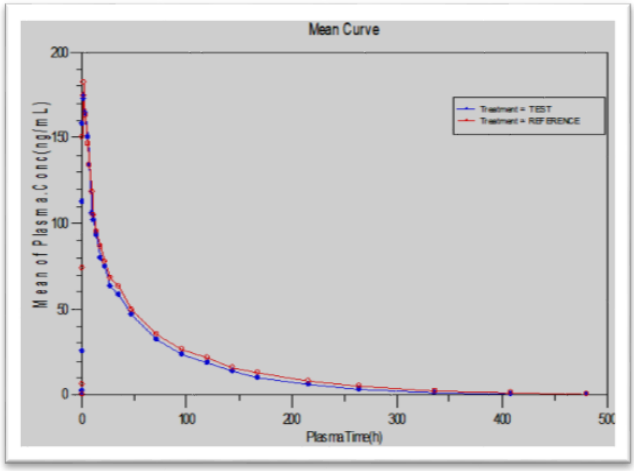

Fig. 1. ***The evolution of PC of MO after treatment with the test and reference products***

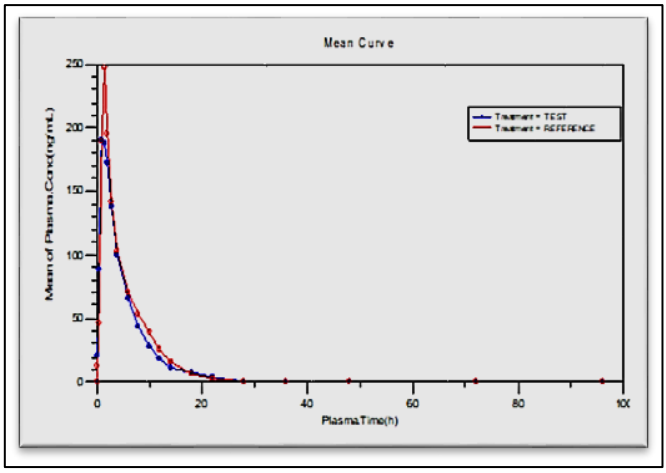

Fig. 2***. Pharmacokinetic parameter values of the tested active molecules (MO and PZ)***

The correlated analysis of clinical, hematological, biochemical, pharmacokinetic, and statistical data highlights compliance, tolerance, and safety of treatment with the generic product Milbenin, positioning it as an interchangeable antihelminthic product in canine therapy, indicated for the control of mixed parasitoses, including dirofilariasis.

Table 2. ***Pharmacokinetic parameter values of the tested active molecules (MO and PZ)***

Parameter	Test product			Reference product		
	Mean	Geo. MEAN	St. Dev	Mean	Geo. nean	St.. Dev
Milbemycin oxime (MO)						
C_{max} (µg/mL)	217.34	202.64	96.696	212.68	204.75	60.462
T_{max} (h)	3.3182	2.6431	2.3832	3.3636	2.8828	2.4358
AUC_{fin} (µg/mL/h)	7316	7022.8	2394.4	8169.6	7801.9	2585.6
AUC_{tot} (µg/mL/h)	7812.7	7525.5	2434.3	8737	8345.4	2758.7
$T_{1/2}$ (h)	62.777	58.876	23.526	66.662	60.334	31.548
MRT (h)	79.437	73.972	31.543	86.142	78.233	40.425
Praziquantel (PZ)						
C_{max} (µg/mL)	274.64	258.75	92.967	304.09	273.94	136.78
T_{max} (h)	2.0909	1.6477	1.7904	2.0455	1.6341	2.0581
AUC_{fin} (µg/mL/h)	1037.1	911.1	635.31	1136	938.94	806.95
AUC_{tot} (µg/mL/h)	1056	930.21	637.13	1149.9	952.65	810.27
$T_{1/2}$ (h)	3.1657	2.817	1.8738	2.8109	2.6182	1.0963
MRT (h)	4.7478	4.2767	2.4741	4.5563	4.215	2.0775

Table 3. ***90% confidence interval values of the primary pharmacokinetic parameters for the test/reference (T/R) ratio of MO and PZ in dogs evaluated in the bioequivalence test***

Substance	Reference 90% CI	Geo mean ratio T/R	Reference 90% CI
C_{max}			
MO	0.87479 - 1.1197	0.989693	0.8-1.25
PZ	0.80184 - 1.1126	0.944543	0.8-1.25
AUC_{last}			
MO	0.8381 - 0.96676	0.900135	0.8-1.25
PZ	0.80655 - 1.1674	0.970344	0.8-1.25
AUC_{tot}			
MO	0.8381 - 0.96676	0.900135	0.8-1.25
PZ	0.80655 - 1.1674	0.970344	0.8-1.25

Chapter 10, "Evaluation of the Therapeutic Efficacy of the Generic Product" presents clinically and coproparasitologically relevant results regarding the high degree of interchangeability and therapeutic efficacy (90%) of the generic product *Milbenin*, used in the treatment of natural infestations with *Toxocara canis* and *Dipylidium caninum* in dogs.

The general conclusions and recommendations formulated based on the results obtained from the five doctoral studies complete the content of the thesis.

The originality and innovative contributions of the thesis summarize the main novelties and original elements derived from the five conducted studies, analyzing the innovative nature of the designs and analyses performed, as well as the contributions made to the diversification and expansion of knowledge in the field.

The bibliography includes a rich set of 193 domain-specific references, containing current and relevant titles appropriate for the documentation and interpretations carried out.

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