Analysis of statistical parameters used in bioequivalence assessment of a novel generic anthelmintic formula for sheep

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Abstract

Bioequivalence testing is one of the essential procedures used for marketing authorisation of veterinary medicinal products.The aim of this study was to implement a minimum set of statistical parameters in the bioequivalence assessment protocol of two anthelmintic formulas based on Triclabendazole (50 mg/mL) and Ivermectin (1 mg /mL), orally administered to sheep. The study can be synthesized to determine the relative bioavailability and bioequivalence of the two products on 36 clinically healthy sheep, following an unicentric, randomized, cross-over, two-sequence, two-treatment and 14-day wash-out study design. Determination of plasma concentrations of Triclabendazole sulfoxide and Ivermectin was made by two rapid, selective high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) methods. According to the implemented protocol, the statistical analysis of the data obtained corroborated a set of descriptive parameters (mean, standard deviation, interval) for the sample of subjects (age, weight) with pharmacokinetic parameters relevant for the active substances (Cmax, AUClast, AUCtot) with additional parameters (% extrapolated AUC, thalf, MRT) and drug safety (adverse events, clinical and laboratory screening and follow-up examinations). For bioequivalence assessment to all the primary pharmacokinetic parameters considered (Cmax, AUClast), a confidence interval of 90% for the ratio of the population means must be calculated. All these pharmacokinetic parameters were planned for analysis using ANOVA, after the data have been transformed (logarithmic transformation). A reference 90% confidence interval of 0.8 – 1.25 was chosen. The bioequivalence can be concluded if the calculated 90% confidence interval around the ratio of means (Test/Reference) using log transformed data falls within the reference acceptance range of 0.8–1.25 for all primary pharmacokinetic parameters of triclabendazole sulfoxide and ivermectin. Finally, the bioequivalence of the two anthelmintic products and, respectively, the possibility of exchanging information between them in the veterinary therapeutic field is determined on the basis of the relevance of the values obtained in the statistical analyzes, especially of the pharmacokinetic parameters.

Keywords: *Bioavailability, Bioequivalence, Ivermectin, Triclabendazole.*

Introduction

In bioequivalence studies, an analytical method approved by the international guides, well characterized and documented, must be used in order to obtain valid results (Cristina and Chirciu, 2010). In this context, we should mention that the main characteristics of a performing and relevant bioanalytical method are selectivity, lower limit of quantification, calibration curve, accuracy, precision and stability (Cristea, 2005; Crivineanu, 2008). The main pharmacokinetic parameters analyzed in drug bioequivalence studies are the area under the curve (AUC), the maximum plasma concentration (Cmax) and the time taken to reach the maximum concentration (Tmax) (Crivineanu, 2008; Palermo-Neto and Righi, 2008). Usually, Asc (0-t) and AUC (0-∞) are determined, but for the study of bioequivalence of the immediate release pharmaceutical forms, AUC (0-τ) is determined next to Cmax and Tmax. (Sargent and Chambers, 2009; Arion et al., 2015). Other additional parameters can be used in bioequivalence studies such as elimination constant (λz), half-time (T1/2) and Cmin. The lower limit of quantification must be equal to 1/20 of Cmax and the

concentrations in the predose must be detectable at 5% or less than Cmax (Vas et al., 2011; Valentina et al., 2007).

The determination of Asc implies that the ratio between the two treatments applied is between 80-125% and in the case of Cmax and Cmin the confidence limits must be between 80 and 125%. However, if the investigated parameters possess very high individual variability, the increase of the confidence interval between 70 and 143% is accepted, this being mentioned in the working protocol, together with a valid pharmacokinetic and pharmacodynamic justification (CVMP Guideline, 2012; Official Journal of the European Union - Directive 2010/63 / EU; International Committee for Harmonization, 2019). Such an increase in the confidence interval will not affect the effectiveness and safety of the product. In the case of antimicrobial and pesticide products, when defining the confidence interval, the risk of developing resistance to these substances will be taken into account (EMA / CVMP / 016/00-Rev.2, 2011; VICH GL52 Bioequivalence; https: / /www.ema.europa).

Statistical analysis allows the assessment of bioequivalence, based on the 90% confidence interval for the ratio between the population geometric means (test/reference) of the investigated parameters. The obtained data must be logarithmically transformed and analyzed in the ANOVA system. With this system, a confidence interval is then calculated for the differences between the two active formulas by logarithmic transformation, which will then be retransformed to the original scale, obtaining the desired ratio.

Regarding the clinical impact of the active substances contained in the two products subjected to the bioequivalence test, we only mention the results of a study focused on evaluating the comparative efficacy of Ivermectin, Levamisol and Albendazole in natural infestations with gastrointestinal nematodes in goats (Aktaruzzaman et al., 2015). According to the results obtained in this study, in Bangladesh are predominantly evolving mixed parasitic infestations (given by *Haemonchus spp., Trichostrongylus spp., Cooperia spp., Oesophagostomum spp., Trichuris spp., Strongyloides spp.*), in which Ivermectin (A-mectin) predominate in Bangladesh, Levamisole (Levavet) and Albendazole (Almex-Vet) have proven to be effective and significantly reducing OPG (eggs per gram feces) in these gastrointestinal nematodes. Of the three products, Levavet has been shown to be more effective in the removal and remission of haematological parameters in goats. At the end of the study, it is estimated that the three anthelmintics have a major therapeutic impact, having ovocidal or egg production inhibiting effects in these gastrointestinal nematodes (Aktaruzzaman et al., 2015; Dupuy et al., 2010; Khalid et al., 2004).

The aim of our study consisted of the statistical analysis of the pharmacokinetic parameters of triclabendazole sulfoxide and ivermectin, in order to evaluate the bioavailability and bioequivalence of a new anthelmintic formula under testing. The main objective of the research was focused on the formulation and testing of this new anthelmintic, with the mention that such a generic product has not been reported yet in ovine therapy.

Materials and methods

Subjects used in the test. The tests were performed on Țurcană sheep from the herd of a commercial micro-farm. According to the inclusion/exclusion criteria, a one-year sample of clinically healthy (no=36), non-lactating and non-lactating sheep, consisting of 26 females and 10 males, was selected. The animals were selected 7 days before the start of the research, based on the inclusion criteria, including the physiological references for clinical and hemato-biochemical health parameters. The sheep were predominantly fed with good quality hay and corn and the water was administered at discretion throughout the testing. The feeding was suspended only in the days

of the administration of the medicinal products and the administration of the water was suspended 2 hours before and after the administration of the medication. In order to justify the size of the sheep sample, we should mention that the procedure for testing the bioequivalence of drugs requires the use of a significant sample of animals of the target species**.**

General scheme of the study. The research protocol consisted of bioequivalence testing of two anthelmintic drug formulas based on Ivermectin and Triclabendazole: the reference product, Fasimec duo 50mg / ml + 1mg / ml oral suspension for sheep (Elanco Animal Health, UK), existing on the market and a novel product, Trimectin 50mg/ml + 1mg/ ml oral suspension for sheep (Pharma VIM Kft., Hungary) tested. The experimental procedure was adapted to the bioequivalence testing of the active molecules contained in the investigated products, as well as to sheep testing as the target species. In accordance with the requirements of the international guidelines, a unicentric, randomized, cross-type test was implemented, with two sequences, two treatments, with a single dose and a 14-day break between them.

In the study 36 healthy clinical sheep were selected, in order to count on at least 30 eligible subjects at the end of the test. During the two periods of the study, the collection of serial blood samples was scheduled, under fasting conditions after the product administration. Venous blood samples (5 mL, on EDTA K3) were collected to evaluate the pharmacokinetic parameters strictly following the time intervals included in the protocol, the specific test times being distributed over a period of 480 hours after administration of the products. Thus, blood samples were collected at the following intervals: before administration (time 0.0) and at 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; 22.0; 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; 480.0 hours after dosing. After harvesting, the samples were centrifuged, at 5000 rpm, for 10 minutes, and the plasma was separated into two tubes. Plasmas were transported and stored at -20 ° C and plasma concentrations of triclabendazole sulfoxide and ivermectin were determined by high performance liquid chromatographic method coupled with mass spectrometry (LC-MS / MS). At the beginning and the end of the test, the main hemato-biochemical parameters necessary for the evaluation of the health status were investigated. The study plan is finalized with the bioequivalence evaluation of the test and reference formulas, based on the analysis of the primary pharmacokinetic parameters (Cmax and ASC0-t) of ivermectin and triclabendazole sulfoxide.

Statistical analysis of pharmacokinetic parameters. In the final assessment of the bioequivalence were included the data obtained in the testing of the entire sheep sample, because none of the 36 subjects was excluded from the test.

Pharmacokinetic parameters were analyzed by a non-compartmental method, for both triclabendazole sulfoxide and ivermectin. The bioequivalence assessment of the primary pharmacokinetic parameters (Cmax and AUClast) also included the quantification of a 90% confidence interval for the Test/Reference module. These pharmacokinetic parameters were determined after logarithmic data transformation, using the ANOVA system.

The reference values for the 90% confidence interval were set in the range 0.8-1.25. At the same time, a series of usual statistical parameters were calculated: arithmetic mean, harmonic mean, geometric mean, SEM, standard deviation, median. In order to compare the values obtained at the Tmax parameter, nonparametric tests were performed, using the non-transformed data (KruskalWallis and Friedman's test) and for the calculation of the average Waiting Time (TMA) and the Half Time (T1/2), the ANOVA system was applied. Pharmacokinetic analyzes were based on the use of Kinetica 5-ThermoLabsystems, USA. (https://www.ema.europa).

In order to evaluate the possible statistical and clinical significance of pharmacokinetic interactions, the variance of the main calculated pharmacokinetic parameters was used, using a linear statistical calculation model for the subject and treatment variables.

Results and discussions

The relevance of the analysis of the plasma samples, prepared during the study was ensured by the LC-MS method, which was previously validated by an authorized laboratory (of the Vim Spectrum Company). The results of the pharmacokinetic analyzes of the plasma samples provided a set of data compliant with the current guidelines.

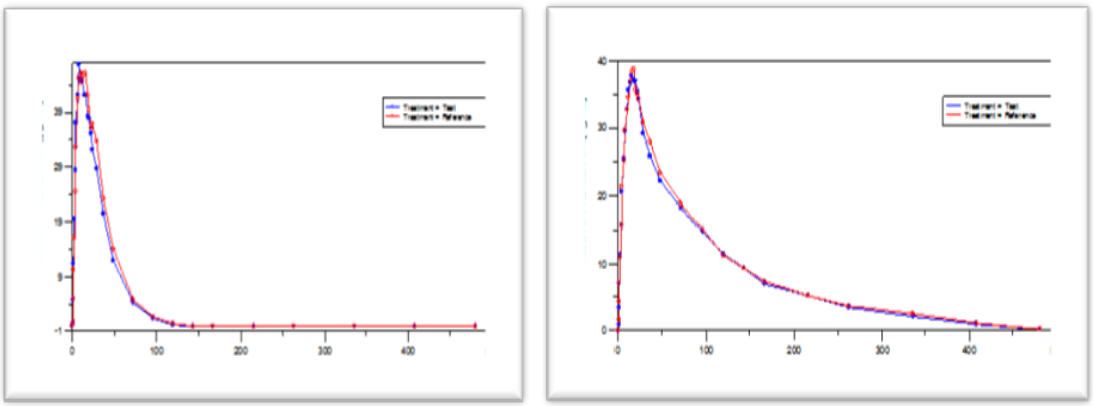
The analysis of these data revealed that between the plasma concentrations recorded at the initial assessments and those obtained at the final evaluations, no differences higher than 20% compared to the average values were found, in the case of 74% of the samples for triclabendazole and 84% of the samples for ivermectin. Equally relevant was the analysis of the distribution of the curves of average plasma concentrations recorded for the two active substances (triclabendazole sulfoxide and ivermectin) in the composition of the test and reference products (Fig. 1).

Framed in the same context, the evolution of average Cmax values revealed reaching levels of 56.0 (+/- 17.1) µg/mL for the test product and 54.4 (+/- 20.1) µg/mL for the reference product, regarding triclabendazole. For ivermectin, however, lower mean Cmax values were found, namely 41.2 (+/- 8.7) ng/mL for the test product and 42.2 (+/- 10.5) ng / mL for the reference product.

There are also noteworthy the data obtained from data processing through the ANOVA system, which revealed the evolution of 90% confidence intervals of the Test/Reference ratio for Cmax (highest concentrations), of 0.98-1.12 for triclabendazole and 0, 92-1.05 for ivermectin.

In the same context, 90% confidence intervals for triclabendazole and ivermectin are also integrated regarding the ratio between the Test and Reference product for AUC, to the last measurable concentration (AUC_{last}), indicating values of 0.88-1.07 for triclabendazole sulfoxide and 0.86-1.06 for ivermectin.

The distribution of mean values and statistics of the pharmacokinetic parameters for triclabendazole sulfoxide and ivermectin are presented in Table 1.



a b

Fig. 1. Curves of mean plasma concentrations of Triclabendazole sulfoxide (a) and Ivermectin (b) during testing of two anthelmintic formulas for sheep

As it appears from the analysis of the obtained results, all the 36 selected subjects were eligible for the pharmacokinetic evaluation and the completion of the testing process. Therefore, all the subjects coressponded with the requirements set out in the experimental protocol and implicitly, with the provision of data for the evaluation of the pharmacokinetics of the active substances introduced in the testing. As a consequence, the products subjected to the bioequivalence test were administered to all the subjects introduced into the study and they were used during two periods, which indicated the achievement of the maximum level of compliance.

We also note that no deviation from the protocol requirements has been recorded and no adverse effects or manifestations have been reported. These achievements were revealed by the predominant classification of the physiological parameters, evaluated at the clinical and paraclinical examinations, in the physiological limits of the species, indicating that the study did not affect the health status of the animals subjected to testing.

The results obtained in the clinical and paraclinical evaluations also highlighted that, compared to the two single doses of triclabendazole and ivermectin, administered orally, as well as the inherent stress associated with the application of the experimental procedures, the investigated sheep showed a high level of tolerance.

It is important to underline the outstanding performance of the LC-MS/MS method used in order to determine the plasma concentrations of triclabendazole sulfoxide and ivermectin, which was initially evaluated and validated in-house.

According to the data obtained at the re-analysis of plasma concentrations, this bioequivalence test met the current legislative standards regarding the validation of the bioanalytical methods (Lainesse et al., 2012; Nation and Sansom, 1994), because the percentage difference between the values obtained at the initial and final analyzes were within the range of +/- 20% of their average, for more than 67% of the tested samples in the case of triclabendazole sulfoxide and ivermectin.

The relevance of the values obtained in the calculation of the primary pharmacokinetic parameters (Cmax, Tmax, AUClast) was also confirmed by the values obtained when quantifying the 90% confidence interval of the T/R ratio of the pharmacokinetic mean, as well as the significance of the difference of the Tmax values recorded in the Friedman și Kruskal-Wallis tests.

Table 1. Descriptive statistical values of the primary, secondary and additional pharmacokinetic parameters, calculated for triclabendazole sulfoxide and ivermectin from the test and reference product composition

Parameter	no	TEST PRODUCT			REFERENCE PRODUCT		
		Mean	GeoMean	St. Dev.	Mean	GeoMean	St. Dev.
Triclabendazole sulfoxide							
C_{max} (ng/ml)	36	55.997	53.409	17.124	54.402	50.808	20.1
T_{max} (h)	36	11.583	10.184	5.6334	11.833	10.957	4.6935
AUC_{last} (ng/ml*h)	36	1655.6	1592.1	443.85	1803.3	1642.9	750.6
AUC_{tot} (ng/ml*h)	36	1702.4	1640.3	445.88	1847.7	1691	755.64
$\%AUC_{extra}$	36	2.9218	2.4573	2.0036	2.8269	2.4212	1.9826
$T_{1/2}$ (h)	36	15.517	15.14	3.7018	15.594	15.234	3.5047
MRT (h)	36	28.334	27.792	5.584	29.388	29.061	4.5714

Ivermectin							
C_{max} (ng/ml)	36	41.22	40.301	8.6637	42.182	41.067	10.462
T_{max} (h)	36	17.5	17.047	4.0107	18.333	17.61	5.6061
AUC_{last} (ng/ml*h)	36	3804.3	3562.4	1295.3	3926.9	3719.3	1310.5
AUC_{tot} (ng/ml*h)	36	4001	3754.4	1338.7	4172.6	3952.9	1389.2
$\%AUC_{extra}$	36	5.0776	4.6054	2.563	5.88	5.3701	2.3993
$T_{1/2}$ (h)	36	84.295	79.574	27.945	91.045	87.29	27.042
MRT (h)	36	118.36	113.94	30.72	124.97	121.44	29.278

Synthetically expressed, the values of the 90% confidence intervals recorded for the T/R ratio of triclabendazole and ivermectin were within the conventional range of drug bioequivalence, comprised between 80 and 125% for all primary parameters, the differences recorded in the Tmax (in the Friedman and Kruskal-Wallis tests) being devoid of statistical significance for both active substances.

Summarizing the above data, we can consider that all the requirements of the current legislation, in the field of drug bioequivalence testing have been fulfilled, in order to establish that the investigated products are bioequivalent. In order to explain the achieved level of bioequivalence, we resort to a detailed presentation of the results obtained in correlation with those reported by other researchers in the field. Therefore, following the evaluation of the bioequivalence on 36 complete data sets and the evolutionary analysis of plasma concentrations, we revealed a high degree of similarity between the primary pharmacokinetic parameters of triclabendazole sulfoxide and ivermectin, determined after oral administration of the test and reference products. Specifically, for triclabendazole sulfoxide the average bioequivalence of the Test/Reference (T/R) ratio was 1.05119 for Cmax and 0.969058 for AUClast, and the 90% confidence intervals (Test / reference ratio) were 98,276 - 112.44% for Cmax and 87,971 - 106.75% for AUClast. We also found similar data in the case of ivermectin, the average bioequivalence being 0.981343 for Cmax and 0.957828 for AUClast, and the 90% confidence intervals of 92.099 - 104.57% for Cmax and 86.26 - 106.36 % for AUClast.

According to the current legislation, if during the course of the bioequivalence studies of the veterinary medical products, the concentration in the predose is less than 5% or equal to 5% of the Cmax value of the tested subject, its values can be included without changes in all measurements and pharmacokinetic calculations (EMA Guide / CVMP / 016/00-Rev.2, 2011). Similar data revealed the analysis of the results obtained in the Kruskal Wallis and Friedmanan tests, which did not show statistically significant differences between the values recorded in the test and reference products, in terms of the evolution of C_{max} and AUC_{last} values. In addition to this, if different distributions and important variations of the statistical data appear in the bioequivalence studies, it is necessary to use novel statistical methods, especially in the category of the non-parametric ones, as the international guides in the field foresee (EMA/CVMP/016/00-Rev.2, 2011; VICH GL52 Bioequivalence; https://www.ema.europa). On the other hand, if the plasma levels of the test product were higher than that of the reference product, the status of the reference formula would be uncertain. In such cases, it is usually necessary to increase the number of animals subjected to the research. We also recall that, in the case of drugs with very variable values, we can accept bioequivalence under the following conditions: if the number of subjects is maximum and

in accordance with the requirements of the ethics committee, if the coefficient of variation is over 30% and if the ratio of the geometric mean is between 0.8 and 1.25.

Synthesizing the data obtained in our study, in comparison with the legal requirements, all the requirements for establishing the bioequivalence of the two products were fulfilled, without the need to supplement the number of animals.

Conclusions

Finally, based on the results obtained in this research, we conclude that the TEST product, Trimectin 50 mg / mL + 1 mg / mL oral suspension for sheep (Pharma VIM Ltd.) is bioequivalent to the REFERENCE product, Fasimec Duo 50 mg / mL + 1 mg / mL oral suspension for sheep (Elanco Europe Ltd.), regarding the speed and degree of absorption of triclabendazole sulfoxide and ivermectin.

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