Veterinary Parasitology 268 (2019) 81–86

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Relationship between pharmacokinetics of ivermectin (3.15%) and its efficacy to control the infestation with the tick *Rhipicephalus* (*Boophilus*) *microplus* in cattle

Santiago Nava[a,*], María V. Rossner[b], Mariana Ballent[c], Atilio J. Mangold[a], Carlos Lanusse[c], Adrián Lifschitz[c]

[a] Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela(INTA EEA Rafaela) and Consejo Nacional de Investigaciones Científicas y Técnicas(CONICET), Rafaela, Santa Fe, Argentina
[b] Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Colonia Benítez(INTA EEA Colonia Benítez), Colonia Benítez, Chaco, Argentina
[c] Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET-CICPBA, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina

ARTICLE INFO

Keywords:
Rhipicephalus microplus
Control
Ivermectin
Plasma concentration

ABSTRACT

This study aimed to determine the relationship between the variation in plasma concentration of ivermectin 3.15% over time and its efficacy against the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*. In addition, a trial was conducted to infer if the application of successive treatments with ivermectin 3.15% could affect its accumulation in cattle. A noticeable variation of ivermectin plasma concentration was observed among the treated heifers. However, these differences did not have a significant effect onthe therapeutic efficacy of the treatment at the end of the trial. No significant differences were observed in the levels of tick infestations between heifers of the treated group; moreover, no significant correlation was detected between the plasma $AUC_{0\text{-}21}$ of ivermectin 3.15% and the cumulative number of ticks of each heifer. Levels of therapeutic efficacy higher than 80% were observed only from day 7 post-treatment, when levels of ivermectin concentration were higher than 8 ng/ml. The lowest values of therapeutic efficacy were observed during the first and the second days post-treatment, when plasma concentrations of ivermectin 3.15% were lower than 8 ng/ml. Viable engorged females were collected from the heifers belonging to the treated group from days 1–5 post-treatment. There was a significant accumulation of the drug after the second dose of ivermectin 3.15%. Ivermectin concentrations in fat biopsies were 366 ng/g (51 days after the first treatment), 275 ng/g (51 days after the second treatment) and 15 ng/g (64 days after the second treatment). These results suggest that applications of successive treatments with ivermectin 3.15% might increase its accumulation in cattle tissues, extending the withdrawal period indicated for the commercial formulation.

1. Introduction

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is a major pest of cattle in tropical and subtropical areas of the world (Jongejan and Uilenberg 2004). This tick and the haemoparasites it transmits impose important constraints on cattle production since they are directly associated with reduced weight gain and milk production, hide damage, mortality, morbidity and control costs, and because tick infestations facilitate the occurrence of screwworm myiasis in cattle (Späth et al., 1994; Reck et al., 2014b). The wide use of chemical acaricides to control ticks is also related to serious problems such as multidrug resistance and accumulation of chemical residues in meat or milk (George et al., 2008; Nari Henrioud, 2011; Guerrero et al., 2012; Reck et al., 2014a; Klafke et al., 2017).

Ivermectin, an endectocide belonging to the avermectin family (Campbell and Benz, 1984), is part of the macrocyclic lactone class. Avermectin endectocides are characterized by their high efficacy against endo- and ectoparasites and long persistence of antiparasitic activity (McKellar and Benchaoui, 1996). Ivermectin long-acting formulations (3.15%) are widely used to control *R.* (*B.*) *microplus* infestation in cattle. The use of ivermectin 3.15% against *R.* (*B.*) *microplus* and its effect on the reproductive parameters of this tick have been evaluated in different countries of the Americas (Arieta-Román et al., 2010; Davey et al., 2010; Lopes et al., 2013; Nava et al., 2015).

* Corresponding author.
E-mail address: nava.santiago@inta.gob.ar (S. Nava).

https://doi.org/10.1016/j.vetpar.2019.03.008
Received 4 December 2018; Received in revised form 23 March 2019; Accepted 30 March 2019

Particularly in Argentina, several commercial formulations of ivermectin 3.15% are officially approved for the control of *R.* (*B.*) *microplus* (http://www.senasa.gob.ar), which demonstrates the importance of this drug in the schemes designed to control the cattle tick. However, the widespread use of ivermectin for tick control has drawbacks, such as the emergence of resistant ticks and helminths of cattle and its long withdrawal period. For, example, the withdrawal period of commercial formulations of ivermectin 3.15% from last treatment until cattle slaughter for meat ranges from 50 to more than 100 days, and its use in dairy cattle inproduction is not recommended.

The pharmacokinetic process, which includes drug absorption, tissue distribution, and biotransformation/elimination pattern, is crucial for the drug to reach its target parasites at sufficient concentrations and over a period long enough to exert a significant effect (Lanusse et al., 2018). In this sense, there is a strong relationship between pharmacokinetics (which determines drug exposure at the parasite location site) and pharmacodynamics, i.e. drug effect (Lanusse et al., 2018). The efficacy of ivermectins is related to systemic drug exposure, which can be evidenced by the comparison of drug concentration profiles in plasma (Gayrard et al., 1999). A high correlation was observed between avermectin concentrations (eprinomectin) recovered in plasma and those measured within the ticks (Lifschitz et al., 2016). Variations in ivermectin plasma concentration are related to different factors, such as target species, body weight, body condition, physiological status, nutritional state, tissue of the host, route of administration and commercial formulation (Gayrard et al., 1999; Lifschitz et al. 1999, 2000, 2007; González Canga et al., 2009). Knowledge about how variations in pharmacokinetic patterns in the target animal species affect the efficacy against a given parasite is necessary for an appropriate design of control methods. Therefore, this work aimed to: I) determine the relationship between plasma concentration of ivermectin 3.15% over time and its therapeutic efficacy against a field strain of the cattle tick *R.* (*B.*) *microplus*; II) infer how the inter-individual variation in plasma concentration of ivermectin 3.15% over time affects the efficacy of tick control; III) verify whether the initial tick load in cattle affects the absorption and persistence of ivermectin 3.15%. Finally, a trial was also performed to determine if the applications of successive treatments with ivermectin 3.15% may affect its accumulation in cattle.

2. Material and methods

2.1. Trial 1: Ivermectin concentrations in plasma and its efficacy for tick control

Twenty 30-month-old Braford heifers were used as experimental animals in the Estación Experimental Agropecuaria Colonia Benitez, Instituto Nacional de Tecnología Agropecuaria (INTA EEA Colonia Benítez), Colonia Benitez (27°20′S, S 58°56′W), Chaco province, northeastern Argentina. These heifers, naturally infested with *R.* (*B.*) *microplus* ticks, were divided into two homogeneous groups(groups I and II) on day 0 on the basis of pre-treatment tick counts. Heifers belonging to group I were treated with a subcutaneous injection of a commercial formulation of ivermectin 3.15% (Bagomectina® 3.15LA AD3E, BIOGÉNESIS BAGÓ S.A, Argentina; lot# 029 A, expiration date 12/2019) at a rate of 1 ml/50 kg of body weight (630 μg/kg) on day 0 (15th March 2017),whereas animals included in group II (control group) were not treated. All heifers had been grazing on the same pasture for more than 6 weeks prior to day 0. Blood samples were taken from the jugular vein from all the treated animals into plastic heparinized tubes (BD Vacutainer®, Becton Dickinson & Company, Argentina) on days 1, 2, 5, 7, 13 and 21 after treatment. The blood was subsequently centrifuged for 10 min at 750*g* and 24 °C. to separate the plasma, which was processed as described in Sections 2.3 and 2.4.

Counts of *R.* (*B.*) *microplus* females (4.5–8.0 mm long) were performed on the left side of each heifer and the whole tail on day 0 (pre-treatment) and on days 1, 2, 5, 7, 13 and 21 post-treatment, to assess the therapeutic efficacy of the treatment with ivermectin 3.15%. The number of half-body collected ticks was multiplied by two for statistical analyses. Prevalence (number of infested hosts /number of examined hosts) and median with first and third quartiles (1Q–3Q) were calculated. Shapiro-Wilk's test was applied to test the normality of the data prior to statistical analysis, which revealed significant deviations from the normal distribution. Thus, the non-parametric Mann-Whitney test was employed to evaluate the statistical significances of the differences in the distribution of *R.* (*B.*) *microplus* numbers between groups. Differences were considered significant at $P < 0.01$. When the counts in both groups were significantly different between them, the corrected efficacy percentage (EP) was calculated with the modified Abbot's formula described by Henderson and Tilton (1955):

$$\text{Corrected EP } \% = \left[\frac{\text{1-n in Co before treatment} * \text{n in T after treatment}}{\text{n in Co after treatment} * \text{n in T before treatment}} \right] * 100$$

where n is the mean number of ticks, T is the treated group (Group I) and Co is the untreated group (Group II).

Tick infestation was compared among animals within group I using Kruskal-Wallis test and a posteriori multiple comparison, according to the procedures described in Conover (1999). The correlation between plasma concentration of ivermectin 3.15% and number of ticks on each heifer was estimated using the Spearman correlation coefficient test (r_s). Statistical analyses were performed using InfoStat software (Universidad Nacional de Córdoba, Argentina).

The effect of ivermectin 3.15% on the reproductive parameters of engorged female ticks collected from the heifers on days 1, 2, 5, 7, 13 and 21 post-treatment was also evaluated. Engorged females collected from both treated and non-treated groups were weighed and kept at 25 °C and 83–86% relative humidity, with a daily photoperiod of 12 h light–12 h dark. Larvae and unhatched eggs were counted as described by Guglielmone et al. (1989). Larval-hatchability rate, the reproductive efficiency index [REI = number of eggs laid/weight of the females in mg (Drummond and Whetstone, 1970)] and the fertility efficiency index [FEI = number of hatched larvae/weight of the females in mg (Aguirre et al., 2005)] were calculated. Differences in the engorged female weight, larval-hatchability rate, REI and FEI values between groups I and II were statistically compared using chi-square test.

2.2. Trial 2: assessment of ivermectin accumulation after successive treatments

A second trial was performed in INTA EEA Colonia Benítez to determine how the applications of two successive treatments with ivermectin 3.15% affect its accumulation in tissues of cattle and the withdrawal period. Ten 20-month-old Braford heifers (kept under the same condition as those described in 2.1) were subjected to two treatments with a subcutaneous injection of a commercial formulation of ivermectin 3.15% (Bagomectina® 3.15LA AD3E, BIOGÉNESIS BAGÓ S.A, Argentina; lot# 029 A, expiration date 12/2019) at a rate of 1 ml/50 kg of body weight (630 μg/kg) on day 0 (10th January 2017) and 35 (14th February 2017). The interval between treatments was determined by adding the residual effectiveness for absolute tick control (28 days in the case of Bagomectina® 3.15LA AD3E) plus a period of 7 days. Heparinised plasma samples were taken from all the heifers as described in Section 3.1 on days 1, 2, 7, 14, 21, 28 and 35 after the first treatment, and on days 1, 2, 7, 16, 21, 28, 35, 43, 50 and 57 after the second treatment, which correspond to days 36, 37, 42, 51, 56, 63, 70, 78, 85 and 92 after the first treatment, respectively. This pattern was chosen considering that the withdrawal period of the used formulation from last treatment until cattle slaughter consumption is 50 days in the case of meat for human. Thus, it allows us to determine the concentration of ivermectin 3.15% in plasma (see methods in Section 2.5) over time in animals subjected to two successive treatments, even

during a period of seven days (day 57 after the second treatment or 92 after the first treatment) after the cessation of the recommended withdrawal period corresponding to the second treatment on day 35.

To determine the profiles of ivermectin 3.15% concentration in cattle tissue, fat samples were taken from three randomly selected animals on days 51 after the first treatment (2nd March 2017, one day after cessation of the recommended withdrawal period corresponding to the first treatment), 51 after the second treatment (6th April 2017, one day after the cessation of the recommended withdrawal period corresponding to the second treatment), and 64 after the second treatment (19th April 2017, 14 days after the cessation of the recommended withdrawal period corresponding to the second treatment). Fat biopsies (approximately 1 g) were taken from the dorsal part of the flank on either side of the midline. The skin and subcutaneous tissue were anesthetized with 2% lidocaine. A wide spectrum antibiotic (penicillin-streptomycin, Estrepto Pendiben® BIOGÉNESIS BAGÓ S.A, Argentina) was applied on the wound. Protocols for animal welfare were designed according to the Animal Welfare Policy (Academic Council Resolution 087/02, approval number 03/16), Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA),Tandil, Argentina.

All animals involved in trials 1 and 2 were subjected to only one treatment with ivermectin prior to day 0, which was applied between 10 and 12 months before the beginning of this study.

2.3. Chemical extraction of ivermectin and derivatization

Ivermectin concentrations in plasma (trials 1 and 2) and fat samples were analyzed following the methodology described by Lifschitz et al. (1999, 2000). The ivermectin chemical extraction process used an aliquot of 0.25 ml of plasma or 0.25 g of fat and 1 ml of acetonitrile (J.T. Baker ®, Center Valley, PA, USA) as solvent. Doramectin (Sigma-Aldrich) was included in each sample as internal standard. The mixture of samples and the solvent were shaken (Multi Tube Vortexer, VWR Scientific Products, West Chester, PA, USA) for 20 (2400 rpm) min and then samples were sonicated (ultrasonic frequency kHz 35) for 10 min (Transsonic 570/H, Laboratory Line Instruments Inc., Melrose Park, IL, USA). Finally, the samples were centrifuged at 2000 x *g* (15 min) and the supernatant was recovered in the khan tubes (Deltalab, 5 ml). In the case of fat samples, the procedure was repeated twice. The supernatant was concentrated to dryness under a stream of nitrogen. The dry residue of ivermectin was derivatized as previously described by De Montigny et al. (1990).

2.4. Chromatographic conditions

Ivermectin concentrations were determined by high-performance liquid chromatography (HPLC) using a Shimadzu 10 A HPLC system with an autosampler (Shimadzu Corporation, Kyoto, Japan). HPLC analysis was performed using a reverse phase C_{18} column (Kromasil, Eka Chemicals, Bohus, Sweden, 5 µm, 4.6 mm × 250 mm) and a mobile phase of 0.2% acetic acid in water/methanol/acetonitrile (1.6/60/38.4) at a flow rate of 1.5 ml/min at 30 °C (Lifschitz et al., 1999). Ivermectin was detected with a fluorescence detector (Shimadzu, RF-10 Spectrofluorometric detector, Kyoto, Japan), reading at 365 nm (excitation) and 475 nm (emission wavelength). Linearity was established to determine the ivermectin concentrations/detector responses relationship. Calibration curves were prepared within the 0.2–80 ng/g range. Experimental concentrations higher than the upper limit of the curve were diluted to be included in the linear range. Calibration curves were established using least squares linear regression analysis and correlation coefficients (r) and coefficient of variations (CV) were calculated. Percentages of ivermectin recovery from plasma and fat were > 70%. The method precision measured with the coefficient of variation was between 0.62 and 8.58%. The limit of quantification was established at 0.2 ng/ml (plasma) and 1 ng/g (fat).

2.5. Pharmacokinetic analyses

A non- compartmental pharmacokinetic analysis was performed with the PK Solutions 2.0 computer software (Ashland, Ohio, USA). The pivotal pharmacokinetics parameters were the area under the concentration vs. time curves (AUC), the peak concentration (C_{max}), the time to peak concentration (T_{max}) and the (elimination) half-life ($T_{½el}$) (Gibaldi and Perrier, 1982). The accumulation of ivermectin after successive treatments was calculated based on the linear-dose kinetic parameters (AUC and Cmax). The partial $AUC_{0\text{-}35}$ and $AUC_{36\text{-}70}$ and the Cmax obtained after both injections of ivermectin were statistically compared. Plasma concentrations and the pharmacokinetic parameters are reported as mean ± standard deviation (SD).

Mean PK parameters were statistically compared using Student´s *t* test. Significant differences among SD were subjected to a non-parametric Mann-Whitney test. The statistical analyses were performed using the Instat 3.0 software (GraphPad Software, CA, US).

3. Results

3.1. Trial 1

The results regarding the efficacy of ivermectin 3.15% against *R.* (*B.*) *microplus* during the first 21 days after treatment are shown in Table 1. The two groups showed a similar level of *R.* (*B.*) *microplus* infestation on day 0 (Mann–Whitney U test, $P > 0.05$).The mean number of ticks of groups I and II on day 0 were 172.2 and 171, respectively. The differences in the level of tick infestation between groups were statistically significant in all post-treatmnet counts ($P < 0.01$). The values of efficacy percentage showed an increasing general trend from day 1 to day 21, with the exception of the count of day 13 which showed a lower efficacy percentage than in the previous count (Table 1). Levels of efficacy higher than 80% were reached from day 7 post-treatment, and the maximum value (96.2%) was observed on day 21 post-treatment (Table 1). Although a natural decrease in tick abundance on animals of group II was observed between days 13 and 21 post-treatment, it was not high enough to affect the results of comparisons.

The effect of ivermectin 3.15% on the reproductive parameters of *R.* (*B.*) *microplus* is shown in Table 2. Engorged females were collected on days 1, 2 and 5 post-treatment in group I, but not on days 7, 13 and 21 post-treatment. On the other hand, engorged females were collected in all counts in group II, except for count on day 13 post-treatment. The comparison of the biological parameters between the engorged females

Table 1
Prevalence (P), median (M) and first and third quartiles (1Q-3Q) of *Rhipicephalus* (*Boophilus*) *microplus* females 4.5–8.0 mm long from the treated heifers with ivermectin 3.15% on day 0 (group I). Group II includes heifers without treatment. The efficacy percentage (EP) in numbers of ticks in the group I in relation to group II is also shown.

Day post-treatment	Group I P (%)	Group I M (1Q-3Q)	Group II P (%)	Group II M (1Q-3Q)	EP (GI-GII)
DPT 0	100	139 (126-214)[a]	100	142 (134-200)[a]	NA
DPT 1	100	71 (54-88)[a]	100	114 (46-136)[b]	31.6
DPT 2	100	66 (52-104)[a]	100	100 (58-148)[b]	33.7
DPT 5	100	52 (44-70)[a]	100	142 (112-160)[b]	58.2
DPT 7	100	14 (12-22)[a]	100	110 (70-156)[b]	85.0
DPT 13	100	29 (28-38)[a]	100	52 (32-73)[b]	46.2
DPT 21	40.0	0 (0-2)[a]	100	21 (13-30)[b]	96.2

Mann–Whitney test. Values within rows not sharing superscripts are significantly different ($P < 0.01$).
NA: not applicable.

Table 2
Values of reproductive parameters (mean ± standard deviation) of *Rhipicephalus* (*Boophilus*) *microplus* engorged females collected on heifers treated with ivermectin 3.15% (Group I) and on heifers belonging to the untreated group (Group II).

	EFW (mg) *		LHR (%)*		REI * †		FEI* †	
	Group I	Group II	Group I	Group II	Group I	Group II	Group I	Group II
DPT 1	200.6 ± 8.5[a] (n:10)	224 ± 12.6[b] (n:10)	90.2 ± 1.3[a]	94.8 ± 1.6[a]	4.2 ± 0.2[a]	7.4 ± 0.3[b]	3.8 ± 0.2[a]	6.7 ± 0.4[b]
DPT 2	122.2 ± 5.6[a] (n:9)	220 ± 10.1[b] (n:10)	93.1 ± 0.9[a]	93.8 ± 1.0[a]	5.1 ± 0.2[a]	7.2 ± 0.4[b]	4.6 ± 0.2[a]	6.5 ± 0.4[b]
DPT 5	116.1 ± 6.9[a] (n:6)	238 ± 13.8[b] (n:10)	96.2 ± 0.7[a]	93.4 ± 1.0[a]	4.2 ± 0.4[a]	8.1 ± 0.6[b]	4.1 ± 0.4[a]	7.7 ± 0.5[b]
DPT 7	NO	228 ± 11.4 (n:8)	–	95.5 ± 0.9	–	7.3 ± 0.3	–	6.6 ± 0.5
DPT 13	NO	NO	–	–	–	–	–	–
DPT 21	NO	219 ± 10.2 (n:7)	–	92.5 ± 1.2	–	7.1 ± 0.5	–	6.5 ± 0.3

*Chi − square test.Values within rows not sharing superscripts are significantly different ($P < 0.01$).
† See definition in materials and methods.
EFW: Engorged female weight. LHR: larval-hatchability rate. REI: Reproductive efficiency index. FEI: fecundity efficiency index. DPT: Day post-treatment. NO: Engorged females not observed on heifers. n: numbers of engorged females evaluated.

obtained in groups I and II from days 1–5 post-treatment was always statistically significant in the cases of engorged female weight, reproductive efficiency index and fecundity efficiency index (Table 2). The reproductive capacity of the engorged tick females fed on the treated heifers between days 1 and 5 post-treatment was significantly reduced ($P < 0.01$), although they produced viable larvae (Table 2). Larval-hatchability rates showed no statistical differences ($P > 0.5$) between groups (Table 2).

The levels of plasma concentration (ng/ml) of ivermectin 3.15% in each heifer belonging to group I are shown in Table 3. A linear increase in plasma concentration of ivermectin 3.15% from day 1 post-treatment (mean value 3.94 ng/ml) to day 13 post-treatment (mean value 24.7 ng/ml was observed (Table 3). The mean Cmax was 21.3 ± 13.9 ng/ml and the mean Tmax was 17.4 ± 4.14 days. Coefficients of variation ranged from 38% (day 1) to 105% (day 13).

The number of *R.* (*B.*) *microplus* females 4.5–8.0 mm long per heifer treated with ivermectin 3.15% is presented in Table 3, together with the cumulative number of ticks and the mean concentration of ivermectin 3.15% obtained for each heifer. Despite the strong variation in plasma concentration of ivermectin 3.15% among heifers, the efficacy percentage of the treatment at the end of the trial was high (more than 90%) and showed no differences among animals belonging to group I (Tables 1 and 3). The statistical comparison performed via Kruskal-Wallis test showed no significant differences in the levels of tick infestations among heifers of group I when all the counts were included in the analysis ($P > 0.9$). No significant correlation was found between the plasma $AUC_{0\text{-}21}$ of ivermectin 3.15% and the cumulative number of ticks of each heifer (Spearman's $p = 0.295$); the correlation between the plasma $AUC_{0\text{-}21}$ and the number of ticks per heifer at the beginning of the trial (day 0) was also non-significant (Spearman's $p = 0.295$).

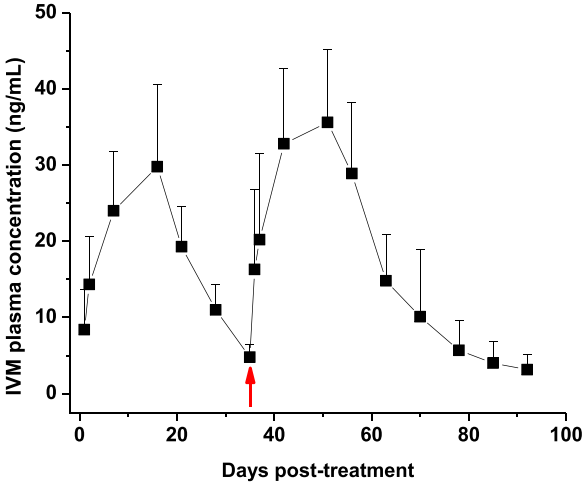

Fig. 1. Mean ivermectin (IVM) plasma concentrations (ng/ml) obtained after the injectable administration of a formulation 3.15% at a dose rate of 630 µg/kg on days 0 and 35. The arrow indicates the date of the second treatment.

3.2. Trial 2

Mean ivermectin 3.15% plasma concentrations measured in the heifers after the two administrations at the recommended dose on days 0 and 35 are shown in Fig. 1. After the first treatment, there was a linear increase from day 1 to day 14 post-treatment and then a noticeable decrease towards day 28 (Fig. 1). The mean Cmax was 30.1 ± 10.5 ng/ml achieved on day 13. A second dose of ivermectin 3.15% was applied on day 35 post-first treatment. A linear increase was also observed from

Table 3
Plasma concentration (ng/ml) of ivermectin 3.15% and number of *Rhipicephalus* (*Boophilus*) *microplus* females 4.5–8.0 mm long (in brackets) per heifer in trial 1 (material and methods).

Heifer identification	Weight (kg)	DPT 0	DPT 1	DPT 2	DPT 5	DPT 7	DPT 13	DPT 21	CNT	$AUC_{0\text{-}21}$
5726	420	NA (148)	4.09 (96)	7.43 (72)	10.8 (52)	10.0 (24)	15.5 (38)	8.18 (0)	430	227
5733	411	NA (130)	4.52 (24)	6.55 (104)	8.92 (86)	9.11 (30)	8.73 (28)	9.86 (2)	404	177
5734	402	NA (138)	5.97 (88)	12.4 (66)	28.1 (66)	29.2 (14)	42.1 (46)	35.8 (0)	418	656
C542	464	NA (246)	1.92 (84)	3.51 (42)	7.07 (46)	8.92 (10)	11.3 (30)	15.9 (0)	458	205
C570	404	NA (126)	1.92 (54)	4.52 (64)	15.9 (52)	14.4 (18)	86.8 (28)	45.1 (2)	344	897
C591	416	NA (104)	2.50 (48)	3.66 (48)	5.24 (34)	5.60 (14)	5.05 (40)	4.87 (2)	290	100
C607	457	NA (396)	6.26 (158)	12.8 (106)	29.7 (44)	33.3 (14)	43.9 (6)	34.0 (0)	724	683
C610	408	NA (140)	4.52 (84)	7.28 (104)	31.1 (44)	20.7 (12)	19.4 (28)	22.1 (0)	412	404
C637	409	NA (80)	3.51 (58)	5.10 (52)	7.45 (70)	6.90 (22)	6.71 (38)	12.1 (2)	322	155
C641	460	NA (214)	4.23 (54)	6.98 (66)	7.80 (92)	8.56 (12)	7.26 (22)	9.29 (0)	460	162
Mean of plasma concentration			**3.94**	**7.02**	**15.2**	**14.7**	**24.7**	**19.7**		
± Standard deviation			1.52	3.28	10.37	9.78	26.01	13.92		

DPT: day post-treatment. CNT: cumulative number of ticks. $AUC_{0\text{-}21}$: individual area under the concentration vs time curve (ng.d/ml) from 0 to 21 days post-treatment. NA: not applicable.

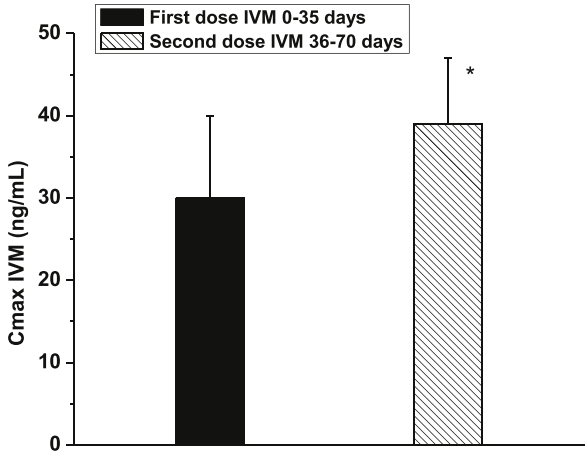

Fig. 2. Peak plasma concentration (Cmax) values obtained after the injectable administration of a commercial formulation of ivermectin (IVM) 3.15% at a dose rate of 630 µg/kg on days 0 and 35. * Values are significantly different at $p < 0.05$.

day 1 to day 16 post-second treatment (day 51 post-first treatment), with a linear decrease towards day 57 post-second treatment (day 92 post-first treatment) (Fig. 1). The highest mean Cmax value (39.0 ± 8.71 ng/ml) was reached on day 15 post-second treatment (Fig. 2). The $AUC_{36\text{-}70}$ (second treatment) was significantly greater than the $AUC_{0\text{-}35}$ (first treatment) (Fig. 3). There was a significant accumulation after the second dose of ivermectin 3.15%. The accumulation ratio was 1.42 for AUC (P: 0.012) and 1.40 for Cmax (P: 0.045). Ivermectin concentrations in fat biopsies were 366 ng/g (51 days post-first treatment), 275 ng/g (51 days post second treatment) and 15 ng/g (64 days post-second treatment).

4. Discussion

The levels of ivermectin plasma concentrations measured during 21 days in trial 1 were similar to those previously reported after the administration of a long-acting formulation (Lifschitz et al., 2007). However, a delayed ivermectin absorption was observed in trial 1 (Tmax at 17 days post-administration) compared to the pioneer formulation (Ivomec Gold®) (Tmax at 9–11 days) (Davey et al., 2000; Lifschitz et al., 2007). The differences in the vehicle composition between the long-acting preparations may explain the different absorption pattern. Moreover, the significantly higher body weight of the

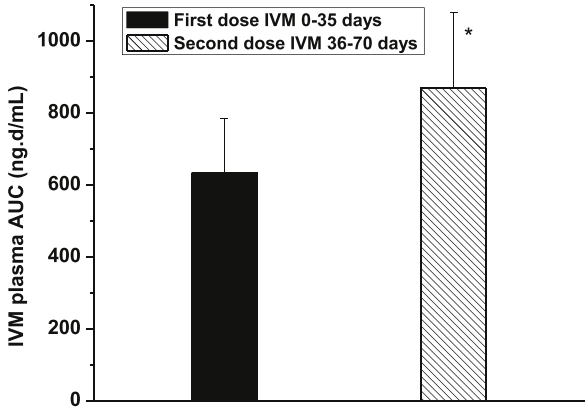

Fig. 3. Systemic exposure measured as the area under the concentration-time curves (AUC) obtained after the injectable administration of a commercial formulation of ivermectin (IVM) 3.15% at a dose rate of 630 µg/kg on days 0 and 35. The bars compare AUC0-35 days and AUC36-70 days. * Values are significantly different at $p < 0.05$.

experimental animals used in trial 1 (above 400 kg, see Table 3) may affect the rate of absorption from the subcutaneous space. A noticeable variation of ivermectin plasma exposure (expressed as ng/ml and $AUC_{0\text{-}21}$) was observed among the heiferstreated with the long-acting preparation (Table 3). The complex process of ivermectin release from the vehicle in the administration site directly influences the drug absorption and may be the main factor of variability among animals. However, these differences did not significantly affect the treatment efficacy at the end of the trial, at least under the conditions and over the study period. In fact, no significant correlation was found either between the plasma $AUC_{0\text{-}21}$ of ivermectin 3.15% and the cumulative number of ticks on each heifer, and between the plasma $AUC_{0\text{-}21}$ and the number of ticks per heifer at the beginning of the trial.

Levels of efficacy percentage higher than 80% were only observed from day 7 post-treatment when levels of ivermectin concentration were higher than 8 ng/ml. The lowest values of efficacy were observed during days 1 and 2 post-treatment, when plasma concentrations of ivermectin 3.15% were lower than 8 ng/ml (Tables 1 and 3). The slow absorption from the subcutaneous space may explain these results. Viable engorged females were also collected from group I heifers from days 1–5 post-treatment. Although the reproductive capacity of these engorged females was somewhat diminished with respect to that of the females collected from the control group (Table 2), they were able to produce viable larvae. Nolan et al. (1981); Davey and George (2002) and Davey et al. (2007) demonstrated that endectocides are less effective against ticks at the final stages of engorgement because there is a lag phase immediately after treatment during which some engorged females do not receive a lethal amount of the drug. The results obtained in this study on days 1 and 2 post-treatment indicate that the efficacy percentage does not reach adequate values in the first days after treatment, when the concentration of ivermectin has still not reached appropriate levels in the blood. In addition, evidence also shows that there is a risk of pasture infestation with engorged tick females that can produce viable progeny. The pharmacokinetic features of the absorption process determined by the vehicle and body composition may directly influence the efficacy outcome during the first days of administration of ivermectin long-acting formulation. All these issues should be considered during the design of plans that include the use of systemic drugs such as ivermectin to control or eradicate ticks.

The sustained concentrations achieved in plasma after the administration of the ivermectin long-acting formulations are relevant to achieve a high effectiveness against ticks (Jackson 1989). The importance of the time of drug exposure above the minimum plasma concentration was demonstrated (Davey et al., 2010; Lifschitz et al., 2016). This information may be useful to establish a pharmacokinetic-pharmacodynamic relationship against *R.(B.) microplus*. A threshold of 8 ng/ml was proposed after the administration of ivermectin long-acting formulation in cattle (Davey et al., 2010). In the present study, the time above 8 ng/ml was calculated for each animal and the average was obtained from the unifested heifers (six animals) and tick infested heifers (four animals) at day 21 post-ivermectin treatment. The mean time above 8 ng/ml was significantly longer in the negative animals. (19.3 ± 1.8 d) compared to the tick positive animals (10.7 ± 8.8 d).

The highest value of efficacy percentage was observed at the end of the trial, on day 21 post-treatment;however absolute control (100% efficacy) was not reached. This result coincides with previous studies performed in other countries, which reported acceptable levels of efficacy of ivermectin 3.15% to control the cattle tick *R. (B.) microplus* but failed to achieve absolute control (Arieta-Román et al., 2010; Lopes et al., 2013). Another concern is related to the efficacy percentage observed on day 13 post-treatment. The efficacy percentage showed an increasing trend from days 1–21 post-treatment, except for the count of day 13, when the efficacy percentage value fell from 85.0% (day 7 post-treatment) to 46.2% (day 13 post-treatment), and then increased to 96.2% on day 21 post-treatment. This phenomenon might be attributed to the accumulation of ticks whose development was affected by the

treatment but not enough to die and detach. Ticks affected by the treatment with ivermectin have a reduced motility and may remain on the cattle for a certain period but without completing development (Errecalde and Mestorino, 2013). Moreover, results of field trials evidenced that some immature ticks fed on cattle treated with ivermectin tend to survive to the young adult stage before succumbing to the treatment (Nolan et al., 1981). Regardless of the results of the count on day 13, the general trend of increasing efficacy values from day 1 to day 21 post-treatment and the absence of viable engorged females after day 5 post-treatment show that the treatment with ivermectin 3.15% achieved acceptable efficacy levels.

Trial 2 evaluated the effect of successive administrations of ivermectin long-acting formulation. The successive treatments with long-acting preparations of ivermectin is a therapeutical tool used by practitioners in the tick-affected areas in South America. Since ivermectin follows a first-order kinetic process in cattle (Lifschitz et al., 2007), dose-dependent pharmacokinetic parameters, such as Cmax and AUC, were used to evaluate the effect of successive ivermectin treatments. A significant accumulation after the second dose of ivermectin 3.15% on day 35 post-first treatment was observed (Figs. 1–3). The accumulation ratio was 1.42 for AUC and 1.40 for Cmax. Regulatory agencies indicate the liver, kidney, muscle and fat as target tissues for evaluation and detection of residual concentrations of ivermectin, with liver and fat being the most important tissues. Ivermectin concentrations were measured in fat, one of the marker tissues. The concentration of ivermectin 3.15% in fat (275 ng/g) measured at day 51 post second treatment, which is the date corresponding to the cessation of the withdrawal period of the used formulation, was also considerable. The limit of maximum residues established by the regulatory agencies may differ among countries. The concentrations of ivermectin in fat measured at 51 days post second treatment may be above the permitted limits established between 40 and 400 ng/g (FAO, 2016; EMA, 2014), whereas ivermectin concentrations at 64 days (15 ng/g) were clearly below the maximum limits of residues for fat. These results show that applications of successive treatments with ivermectin 3.15% may increase its accumulation in cattle tissues, extending the withdrawal period indicated for the commercial formulation. This fact becomes relevant not only from a public health perspective but also for meat commercialization, particularly if a hypothetical scenario of more than two or three successive treatments with ivermectin 3.15% is considered. An international harmonization related to the residues of veterinary drugs is necessary to ensure food safety in the international meat trade.Therefore, there is an evident need to use this type of drugs in treatment schemes that aim not only to achieve therapeutic efficacy but also to avoid residues in tissues of the cattle intended for slaughter. Similar studies taking as target other chemical groups with long withdrawal period officially approved for use as acaricides for cattle should be performed.

Acknowledgements

We are grateful to César Ariel Cingolani for its help in taking fat samples. This study was supported by INTA, Asociación Cooperadora INTA Rafaela, Asociación Cooperadora INTA Colonia Benítez, Agencia Nacional de Promoción Científica y Tecnológica (PICT-2015-0550, PICT-2016-1140) and CONICET, all from Argentina. The revision of English style by Jorgelina Brasca is greatly acknowledged.

References

Aguirre, D.H., Gaido, A.B., Cafrune, M.M., Castelli, M.E., Mangold, A.J., Guglielmone, A.A., 2005. Eprinomectin pour-on for control of *Boophilus microplus* (Canestrini) ticks (Acari: Ixodidae) on cattle. Vet. Parasitol. 127, 157–163.

Arieta-Román, R.J., Rodriguez-Vivas, R.I., Rosado-Aguilar, J.A., Ramírez-Cruz, G.T., Basto-Estrella, G., 2010. Persistencia de la eficacia de dos lactonas macrocíclicas contra infestaciones naturales de *Rhipicephalus* (*Boophilus*) *microplus* en bovinos del trópico mexicano. Rev. Mex. Cienc. Pecu. 1, 59–67.

Campbell, W.C., Benz, G.W., 1984. Ivermectin: a review of efficacy and safety. J. Vet. Pharmacol. Ther. 7, 1–16.

Conover, W.J., 1999. Practical Nonparametric Statistics. John Wiley & Sons, Inc., New York, pp. 592.

Davey, R.B., George, J.E., 2002. Efficacy of macrocyclic lactone endectocides against *Boophilus microplus* (Acari: Ixodidae) infested cattle using different pour-on application treatments regimens. J. Med. Entomol. 39, 763–769.

Davey, R.B., Miller, J.A., George, J.E., Klavons, J.A., 2007. Efficacy of a single doramectin injection against adult female *Boophilus microplus* (Acari: Ixodidae) in the final stages of engorgement before detachment. J. Med. Entomol. 44, 277–282.

Davey, R.B., Pound, J.M., Miller, J.A., Klavons, J.A., 2010. Therapeutic and persistent efficacy of a long-acting (LA) formulation of ivermectin against *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) and sera concentration through time in treated cattle. Vet. Parasitol. 169, 149–156.

De Montigny, P., Shim, J.S., Pivnichny, J.V., 1990. Liquid chromatographic determination of ivermectin in animal plasma with trifluoroacetic anhydride and N-methylimidazole as the derivatization reagent. J. Pharm. Biomed. Anal. 8, 507–511.

Drummond, R.O., Whetstone, T.M., 1970. Oviposition of the gulf coast tick. J. Econ. Entomol. 63, 1547–1551.

EMA, 2014. European Public MRL Assessment Report (EPMAR): Ivermectin. EMA/CVMP/294840/2014.

Errecalde, J., Mestorino, N., 2013. Terapéutica de la Ectoparasitosis. In: Nari, A., Fiel, C. (Eds.), enfermedades parasitarias con importancia clínica y productiva en rumiantes: fundamentos epidemiológicos para su diagnóstico y control. Editorial Hemisferio Sur, Buenos Aires pp. 605-456-638.

FAO, 2016. Ivermectin Residue Monograph. Residue Evaluation of Certain Veterinary Drugs. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 81st meeting 2015. FAO JECFA Monographs 18.

Gayrard, V., Alvinerie, M., Toutain, P.L., 1999. Comparison of pharmacokinetic profiles of doramectin and ivermectin pour-on formulations in cattle. Vet. Parasitol. 81, 47–55.

George, J.E., Pound, J.M., Davey, R.B., 2008. Acaricides for controlling tick on cattle and the problem of acaricide resistance. In: Bowman, A.S., Nuttall, P. (Eds.), Ticks: Biology, Diseases and Control. Cambridge University Press, Cambridge, pp. 408–423.

Gibaldi, M., Perrier, D., 1982. Pharmacokinetics. In: Revised and Expanded, 2nd edn. Marcel Dekker, Inc., New York, USA.

González Canga, A., Sahagún Prieto, A.M., Diez Liébana, M.J., Martínez, N.F., Sierra Vega, M., García Vieitez, J.J., 2009. The pharmacokinetics and metabolism of ivermectin in domestic animal species. Vet. J. 179, 25–37.

Guerrero, F.D., Lovis, L., Martins, J.R., 2012. Acaricide resistance mechanisms in *Rhipicephalus* (*Boophilus*) *microplus*. Rev. Bras. Parasitol. Vet. 21, 1–6.

Guglielmone, A.A., Mangold, A.J., Aguirre, D.H., Gaido, A.B., De Olsen, A.A., 1989. The effect of infection by *Babesia* sp. on some biological parameters of engorged females of *Boophilus microplus*. Folia Parasitol. 36, 1–6.

Henderson, C.F., Tilton, E.W., 1955. Tests with acaricides against the brow wheat mite. J. Econom. Entomol. 48, 157–161.

Klafke, G.M., Webster, A., Dall´Agnol, B., Pradel, E., Silva, J., de La Canal, L.H., Becker, M., Osório, M.F., Mansson, M., Barreto, R., Scheffer, R., Souza, U.A., Corassini, V.B., dos Santos, J., Reck, J., Martins, J.R., 2017. Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul state, southern Brazil. Ticks Tick-borne Dis. 8, 73–80.

Lanusse, C., Canton, C., Virkel, G., Alvarez, L., Costa-Junior, L., Lifschitz, A., 2018. Strategies to optimize the efficacy of anthelmintic drugs in ruminats. Trend. Parasitol. 8, 664–682.

Lifschitz, A., Virkel, G., Pis, A., Imperiale, F., Sánchez, S., Alvarez, L., Kujanek, R., Lanusse, C., 1999. Ivermectin disposition kinetics after subcutaneous and intramuscular administration of an oil-based formulation to cattle. Vet. Parasitol. 86, 203–215.

Lifschitz, A., Virkel, G., Sallovitz, J., Sutra, J.F., Galtier, P., Alvinerie, M., Lanusse, C., 2000. Comparative distribution of ivermectin and doramectin to parasite location tissues in cattle. Vet. Parasitol. 87, 327–338.

Lifschitz, A., Virkel, G., Ballent, M., Sallovitz, J., Imperiale, F., Pis, A., Lanusse, C., 2007. Ivermectin (3.15%) long-acting formulations in cattle: absorption pattern and pharmacokinetic considerations. Vet. Parasitol. 147, 303–310.

Lifschitz, A., Nava, S., Mangold, A.J., Imperiale, F., Ballent, M., Canevari, J., Lanusse, C., 2016. Eprinomectin accumulation in *Rhipicephalus* (*Boophilus*) *microplus*: pharmacokinetic and efficacy assessment. Vet. Parasitol. 215, 11–16.

Lopes, W.D.Z., Teixeira, W.F.P., Matos, L.V.S., Felippelli, G., Cruz, B.C., Maciel, W.G., Buzzulini, C., Fávero, F.C., Soares, V.E., Oliveira, G.P., da Costa, A.J., 2013. Effects of macrocyclic lactones on the reproductive parameters of engorged *Rhipicephalus* (*Boophilus*) *microplus* females detached from experimentally infested cattle. Exp. Parasitol. 135, 72–78.

McKellar, Q., Benchaoui, H., 1996. Avermetins and milbemycins. J. Vet. Pharmacol. Ther. 19, 331–351.

Nari Henrioud, A., 2011. Towards sustainable parasite control practices in livestock production with emphasis in Latin America. Vet. Parasitol. 180, 2–11.

Nava, S., Mangold, A.J., Canevari, J.T., Guglielmone, A.A., 2015. Strategic applications of long-acting acaricides against *Rhipicephalus* (*Boophilus*) *microplus* in northwestern Argentina, with an analysis of tick distribution among cattle. Vet. Parasitol. 208, 225–230.

Nolan, J., Schnitzerling, J., Bird, P., 1981. Evaluation of the potential systemic slow release chemical treatments for control of the cattle tick (*Boophilus microplus*) using ivermectin. Aust. Vet. J. 57, 493–497.

Reck, J., Klafke, G.M., Webster, A., Dall´Agnol, B., Scheffer, R., Souza, U.A., Corassini, V.B., Vargas, R., Santos, J.S., Martins, J.R., 2014a. First report of fluazuron resistance in *Rhipicephalus microplus*: a field tick population resistant to six classes of acaricides. Vet. Parasitol. 201, 128–136.

Reck, J., Marks, F.S., Rodrigues, R.O., Souza, U.A., Webster, A., Leite, R.C., Gonzalez, J.C., Klafke, G.M., Martins, J.R., 2014b. Does *Rhipicephalus microplus* tick infestation increase the risk for myiasis caused by *Conchliomyia hominivorax* in cattle? Prev. Vet. Med. 113, 59–62.

Späth, E.J.A., Guglielmone, A.A., Signorini, A.R., Mangold, A.J., 1994. Estimación de las pérdidas económicas directas producidas por la garrapata *Boophilus microplus* y las enfermedades asociadas en la Argentina. [1ra] parte. Therios 23, 341–360.