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Integrated assessment of ivermectin pharmacokinetics, efficacy against resistant *Haemonchus contortus* and P-glycoprotein expression in lambs treated at three different dosage levels

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ABSTRACT

The main goals of the current work were: (a) to assess the ivermectin (IVM) systemic exposure and plasma disposition kinetics after its administration at the recommended dose, x5 and x10 doses to lambs, (b) to compare the clinical efficacy of the same IVM dosages in lambs infected with an IVM-resistant isolate of *Haemonchus contortus*, and (c) to assess the expression of the transporter protein P-glycoprotein (P-gp) in *H. contortus* recovered at 14 days after administration of the IVM dose regimens. There were two separated trials where IVM was administered either subcutaneously (SC, Experiment I) or intraruminally (IR, Experiment II). Each experiment involved twenty-four (24) lambs artificially infected with a highly resistant *H. contortus* isolate. Animals were allocated into 4 groups ($n = 6$) and treated with IVM at either 0.2 (IVM_{x1}), 1 (IVM_{x5}) or 2 mg/kg (IVM_{x10}). Plasma samples were collected up to 12 days post-treatment and analysed by HPLC. An untreated-control Group was included to assess the comparative anthelmintic efficacy of the different treatments. The level of expression of Pgp in *H. contortus* specimens obtained from lambs both untreated and IR treated with the different IVM doses was quantified by real time PCR. Parametric and non-parametric tests were used to compare the statistical significance of the results ($P < 0.05$). After the SC treatment, the IVM plasma area under the concentration–time curve (AUC_{0-LOQ}) increased from 41.9 (IVM_{SCx1}) up to 221 (IVM_{SCx5}) and 287 (IVM_{SCx10})ng.day/mL and after the IR treatment from 20.8 (IVM_{IRx1}) up to 121 (IVM_{IRx5}) and 323 (IVM_{IRx10})ng.day/mL. Dose-adjusted AUC_{0-LOQ} and Cmax were similar among doses, demonstrating dose proportionality for IVM after both SC and IR administration at the three different doses. The efficacies against resistant *H. contortus* after the SC treatment were 42% (IVM_{SC1}), 75% (IVM_{SCx5}) and 75% (IVM_{SCx10}). However, the IR IVM treatment reached clinical efficacies ranging from 48% (IVM_{IRx1}) up to 96% (IVM_{IRx5}) and 98% (IVM_{IRx10}). None of the IR IVM treatments increased the expression of P-gp in adult *H. contortus* at 14 days post-treatment compared to samples collected from the untreated control group. An enhanced

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parasite exposure of the drug at the abomasum may explain the improved efficacy against this recalcitrant *H. contortus* isolate observed only after the IR administration at 5- and 10-fold the IVM therapeutic dosage.

1. Introduction

Ivermectin (IVM) was the first macrocyclic lactone introduced as an antiparasitic drug in 1981 (Campbell, 1989). IVM is effective against endo- and ecto-parasites in a wide variety of hosts, including cattle, sheep, goats, dogs, swine and horses, as well as domesticated wild animals. A large body of scientific literature has been devoted to the pharmacology properties of the macrocyclic lactones, including detailed pharmacokinetic data (Campbell, 1989; Vercruysse and Rew, 2002) in different animals species. However, there is a lack of basic pharmacokinetic information related to drug absorption and dose proportionality studies in target animal species. Dose proportionality occurs when increments in the administered dose are accompanied by a proportional increment in exposure, measured as either the area under the concentration vs time curve (AUC) or the peak plasma concentration (Cmax). Dose proportionality is a desirable property as it makes predicting the effects of dose adjustments easier, although in practice this is not a global property as it usually applies only to a certain dose range (Hummel et al., 2008). The assessment of dose proportionality is of great clinical importance for predicting the consequences of rational dose adjustments. Recent work demonstrated a non dose-proportionality on the gastrointestinal absorption of albendazole (ABZ) in nematode infected lambs, where increasing the ABZ dose (5- to 9-fold) was clearly associated with enhanced plasma ABZ metabolites exposure (Alvarez et al., 2012), which cannot be explained only by a dose proportionality relationship. The enhanced systemic exposure achieved after ABZ treatments at the highest doses correlated with significant increment in drug efficacy against a resistant *Haemonchus contortus* isolate (Barrère et al., 2012). As far as we know, there are no IVM dose proportionality studies undertaken in sheep.

The impact of increasing the IVM dosage levels on its systemic exposure and in the resultant efficacy against IVM-resistant nematodes in ruminants remains unclear. However, it seems that the transcuticular diffusion is the main route of access for different lipophillic substances, including IVM in gastrointestinal nematodes (Cross et al., 1998; Alvarez et al., 2007). Two major determinants of the rate of transfer across the nematode cuticle are drug lipophilicity (Mottier et al., 2003) and concentration gradient (Mottier et al., 2006). Thus, a higher parasite drug exposure induced by a dose increment, may be assessed as a strategy to kill resistant parasites. For instance, Várady et al. (1996) reported efficacies for IVM against *Oesophagostomum dentatum* in pigs of 88.7, 96.1 and 99.6% after its administration at 0.15, 0.3 or 0.6 mg/kg, respectively. This upward trend in favour of the highest IVM doses could be explained by a higher parasite drug exposure related to the administered dose.

The therapeutic response to an increased anthelmintic dose depends on the genetic diversity in the parasite population being exposed to the drug (Prichard, 2001). Evidence derived from parasitic nematodes and the free living nematode *Caenorhabditis elegans* suggest that the permeability glycoprotein (P-gp) and other ATP-binding cassette (ABC) transporters may be involved on IVM resistance (Molento and Prichard, 1999; Kerboeuf et al., 2003; Prichard and Roulet, 2007; James and Davey, 2009; Ardelli and Prichard, 2013). P-gp is a multidrug membrane transporter which acts as an efflux pump to transport hydrophobic compounds, such as drugs/metabolites, across cell membranes (Higgins, 1992). The activation of this defense mechanism in mammals and nematodes is often observed as changes in expression levels (Chin et al., 1990). Thus, the expression level could be used as an indicator of treatment response of a resistant nematode isolate.

The main goals of the current work were: (a) to assess the IVM systemic exposure and disposition kinetics after its subcutaneous (SC) and intraruminal (IR) administrations at the recommended, x5 and x10 doses to lambs, (b) to compare the clinical efficacy of the same IVM dosages in lambs infected with an IVM-resistant isolate of *H. contortus*, and (c) to assess the expression of P-gp in *H. contortus* collected at 14 days after the IR IVM treatments.

2. Materials and methods

2.1. Animals

Parasite free Corriedale lambs (6–7 months old, 25.2 ± 5.6 kg), artificially infected (trial day −41) with an IVM-resistant *H. contortus* isolate (7.000 L_3/animal) were involved in the current trial. The isolate was from a sheep Experimental Unit (Reserva 8, Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina) with a parasite control programme based on the intensive use of anthelmintics over many years. The use of IVM several times a year over many years had been documented until 1997 (Entrocasso C., personal communication). It had been previously found that IVM failed to control this *H. contortus* strain. Efficacies of 80% (FECRT, SC treatment, Entrocasso et al., 2008), 0% (controlled test, SC treatment, Lifschitz et al., 2010), 40% (controlled test, IR treatment, Lloberas et al., 2012), 20% (controlled test, IR treatment, Lloberas et al., 2013) and 0% (controlled test, IR treatment, Lloberas et al., in press), were reported for this isolate after IVM treatment (0.2 mg/kg). Forty days after infection (trial day −1), all lambs were checked for faecal egg counts (epg), ear tagged and their individual body weights were

recorded. Animals were housed in a stall without access to grass and fed with total mixed grain-based ration (Ovino® TandilCoop, Tandil, Argentina) during the experiment and for 10 days before nematode infection. All the animals had free access to water. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar).

2.2. Experimental design

2.2.1. Experiment I: SC treatment

Twenty four lambs with an average of 8293 ± 2714 epg counts (trial day −1) ranging from 4600 to 13,400 epg counts, were randomly allocated into four experimental groups ($n = 6$). Experimental animals received the following treatments (trial day 0): **IVM_{SCx1}**, animals were treated with IVM (*Ivomec*®, IVM 1%, Merial, Argentina) by the SC route at the therapeutic dose of 0.2 mg/kg; **IVM_{SCx5}**, IVM was administered by the SC route at the 1.0 mg/kg dose (dose x5); **IVM_{SCx10}**, IVM was administered by the SC route at the 2.0 mg/kg dose (dose x10); and **Control**, animals were kept without treatment as controls.

2.3. Experiment II: IR treatment

Twenty four lambs with an average of 6054 ± 4594 epg counts (trial day −1) ranging from 1120 to 18,180 epg counts, were randomly allocated into four experimental groups ($n = 6$). Experimental animals received the following treatments (trial day 0): **IVM_{IRx1}**, animals were treated with IVM (*Ivermectina 0.2 oral*®, IVM 0.2%, Rosenbusch, Uruguay) by the IR route at the therapeutic dose of 0.2 mg/kg; **IVM_{IRx5}**, IVM was administered by the IR route at the 1.0 mg/kg dose (dose x5); **IVM_{IRx10}**, IVM was administered by the IR route at the 2.0 mg/kg dose (dose x10); and **Control**, animals were kept without treatment as controls.

2.4. Pharmacokinetic study

Blood samples (3 mL) were taken from the jugular vein using 10 mL heparinised Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA), before administration (time 0) and at 2, 4, 8 and 12 h and 1, 2, 3, 5, 7, 9, 12 and 14 days after the SC administration of IVM. A similar sampling schedule was used after the IR administration, with the difference that the first three samples post-treatment were obtained at 3, 6 and 12 h. Plasma was separated by centrifugation at 2000 × *g* for 15 min, placed into plastic tubes and frozen at −20 °C until analysis by high performance liquid chromatography (HPLC). In both experiments, any unusual behaviour such as diminishing food consumption, ataxia or prostration, was recorded as a potential sign of toxic effects induced by treatment.

2.5. Analytical procedures

Sample cleanup and derivatization: The extraction of IVM (22,23-dehydro-avermectin B1a), from spiked and experimental plasma samples was carried out following a well established technique (Alvinerie et al., 1993, slightly modified by Lifschitz et al., 1999). Namely, 1 mL aliquot of plasma sample was combined with 10 ng of the internal standard compound (abamectin) and then mixed with 1 mL of acetonitrile–water (4:1). After mixing for 20 min, the solvent–sample mixture was centrifuged at 2000 *g* during 15 min. The supernatant was manually transferred into a tube that was then placed on the appropriate rack of an Aspec XL sample processor (Gilson, Villiers Le Bel, France). The supernatant was injected to a Supelclean LC_{18} cartridge (Supelco, Bellfonte, PA, USA), previously conditioned by passing 2 mL methanol and 2 mL deionized water. The cartridge was flushed with 1 mL of water and 1 mL of water/methanol (4:1). The compounds were eluted with 1.5 mL of methanol and concentrated to dryness under a stream of nitrogen. The re-suspension was done with 100 μL of a solution of N-methylimidazole (Sigma Chemical, St. Louis, MO, USA) in acetonitrile (1:1) (De Montigny et al., 1990). Derivatization was initiated adding 150 μL of trifluoroacetic anhydride (Sigma Chemical, St Louis, MO, USA) solution in acetonitrile (1:2). After completion of the reaction (<30 s), an aliquot (100 μL) of this solution was injected directly into the chromatograph.

HPLC analysis: IVM concentrations were determined by HPLC using a Shimadzu 10 A HPLC system with autosampler (Shimadzu Corporation, Kyoto, Japan). HPLC analysis was undertaken using a reverse phase C_{18} column (Phenomenex, 5 μm, 4.6 mm × 250 mm) and an acetic acid 0.2% in water/methanol/acetonitrile (3.8/40/56.2) mobile phase at a flow rate of 1.5 mL/min at 30 °C. IVM was detected with a fluorescence detector (Shimadzu, RF-10 Spectrofluorometric detector, Kyoto, Japan), reading at 365 nm (excitation wavelength) and 475 nm (emission wavelength). IVM concentrations were determined by the internal standard method using the Class LC 10 Software version 1.2 (Shimadzu Corporation, Kyoto, Japan). The peak area ratios were considered to calculate the IVM concentrations in spiked (validation) and experimental plasma samples. There was no interference of endogenous compounds in the chromatographic determinations. The solvents (Baker, Phillipsburg, NJ, USA) used during the extraction and drug analysis were HPLC grade. A complete validation of the analytical procedures used for extraction and quantification of IVM was performed before starting analysis of the experimental samples obtained during the pharmacokinetic trial. Calibration curves in the range between 0.1–5 ng/mL and 5–100 ng/mL were prepared for each compound. Calibration curves were established using least squares linear regression analysis and correlation coefficients (r) and CV calculated. Linearity was established to determine the IVM concentrations/detector response relationship. Percentages of IVM recovery from plasma were obtained in the range between 0.1 and 50 ng/mL. The inter-assay precision of the extraction and chromatography procedures was estimated by processing replicate aliquots ($n = 4$) of pooled sheep plasma samples containing known IVM concentrations (0.2, 10 and 50 ng/mL) on different working days. The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV <20% an accuracy of ±20% and an absolute

recovery ≥70%. The LOQ obtained for IVM was 0.1 ng/mL. Values below LOQ were not included in the pharmacokinetic analysis. The linear regression lines for IVM showed correlation coefficients ≥0.998. Mean absolute recovery percentages for concentrations ranging between 0.2 and 50 ng/mL were 82%. The inter assay precision of the analytical procedures obtained after HPLC analysis of IVM on different working days showed CV between 4.36% and 6.91%. The LOQ was established at 0.1 ng/mL.

2.6. Pharmacokinetic analysis of the data

Non-compartmental pharmacokinetic analysis for the plasma concentration versus time curves for IVM for each individual animal after the different treatments were conducted using the PK Solution 2.0 (Summit research services, CO, USA). The peak concentration (Cmax) and time to peak concentration (Tmax) were displayed from the plotted concentration–time curve of IVM. The area under the concentration–time curve (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope (β). The elimination ($T½$el) and absorption ($T½$ab) half-lives were calculated as $\ln 2/\beta$ and $\ln 2/k$, respectively. The mean residence time (MRT) was determined as AUMC/AUC (Perrier and Mayersohn, 1982) where AUMC is the area under the curve of the product of time and the plasma drug concentration vs. time from zero to infinity (Gibaldi and Perrier, 1982), and AUC is as defined above.

2.7. Efficacy study

Direct adult nematode counts obtained from animals in treated and control experimental groups ($n=6$ for each group), were determined 14 days after the SC or IR treatments, according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Wood et al., 1995). The efficacy of each anthelmintic treatment was determined by the comparison of worm burdens in treated versus untreated animals. The following equation expresses the percentage of efficacy (%E) of a drug treatment against adult *H. contortus* in a single treatment group (T) when compared with an untreated control (C):

$$\%E = \frac{\text{Mean of } S \text{ in } C - \text{Mean of } S \text{ in } T}{\text{Mean of } S \text{ in } C} \times 100$$

Efficacy was calculated on the arithmetic means, based on analysis that arithmetic means better represent estimates of efficacy than geometric means (McKenna, 1997).

Additionally, the anthelmintic efficacy of the treatments was evaluated by the faecal egg count reduction test (FECRT), calculated according to the formula (Coles et al., 1992):

$$\text{FECRT}(\%) = 100 \times \left(1 - \frac{\text{Ct}}{\text{Cc}}\right),$$

where Ct is the arithmetic mean epg counts in the treated group at 14 days post-treatment and Cc is the arithmetic mean epg counts in the untreated control group at 14 days post-treatment. The 95% confidence intervals were calculated as reported by Coles et al. (1992).

2.8. Complementary P-glycoprotein expression study

2.8.1. Sampling

Samples (adult *H. contortus*) collected from lambs sacrificed at 14 days post-treatment (Experiment II, IR treatment, groups Control, IVM_{IRx1} and IVM_{IRx10}), were obtained for P-gp expression studies. From the abomasum of each animal, pooled adult specimens of *H. contortus* were rapidly recovered, gently washed in saline solution at 4 °C and immediately frozen in vials placed in liquid nitrogen to study the nematode P-gp expression.

2.8.2. Isolation of RNA and reverse transcription

Samples for total RNA (tRNA) extraction were isolated from frozen *H. contortus* specimens recovered from experimental animals sacrificed at 14 days post- IVM treatment. The tRNA from parasites was extracted by Trizol reagent® (Life technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. The RNA integrity and DNA contamination were assessed by the 18S and 28S band intensity ratio after 1.5% agarose gel electrophoresis visualized by SYBR safe staining (Life technologies, Carlsbad, CA, USA). Total RNA purity and concentration were determined spectrophotometrically by recording the absorbance at 260 and 280 nm after a 1:500 sample dilution in RNase-free water. In all samples, the 260/280 ratio (purity) was higher than 1.8. The reverse transcription was performed with 2 μg of total RNA in a final volume of 20 μL, using the High Capacity cDNA Reverse Transcription Kit (Life technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The reaction was performed in a thermocycler for 10 min at 25 °C, 120 min at 37 °C and finally 5 s at 85 °C for enzyme inactivation. The tRNA and cDNA were stored at −70 °C for future use.

2.8.3. Reference and target genes

The primer sequences of the reference and target genes used in the current work and their corresponding GenBank/bibliographic reference, GC% content and amplicon sizes are listed in Table 1. The NCBI (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) and Ensembl (Ensembl Genome Browser [http://www.ensembl.org/index.html]) databases were used to search for available *H. contortus* gene sequences to design primers using the Primer Express software version 2.0 (Applied Biosystems, Foster City, CA, USA) and synthesized by Invitrogen (Carlsbad, CA, USA). The amplicon specificity of the primer pair was tested by a Blast analysis against the genomic NCBI database.

2.8.4. Quantitative real-time PCR (qPCR)

The relative expression levels of P-gp were carried out in an ABI Prism 7500 Real Time PCR System (Applied Biosystems SA). The reaction mixture included 10 μL of PCR power SYBR Green Master Mix 2X (Applied Biosystems SA), 2 μL of each primer set, 1 μL of cDNA diluted 1:250 and 7 μL of water to obtain a final volume of 20 μL. The amplification reaction was performed in a 96-well

Table 1
GenBank accession number, primer sequences and amplicon size of primer sets used for qPCR.

Gen symbol	Primer sequence (5′→3′)	GC (%)	Amplicon size (bp)	GenBank accession No./Reference
H. contortus				
ACT	F: GCTCCCAGCACGATGAAAA R: CACCAATCCAGACAGAGTATTTGC	54 46	66	DQ_080917
P-gp homolog	F: CGGCAGCAGATCTCATGGT R: TCGGTTAGACGAGCTGTGAGATT	58 48	63	AF_003908

ACT: actin; bp: base pair; P-gp: P-glycoprotein.

plate. The qPCR was carried out using the following thermal profile: 2 min at 50 °C followed by 10 min at 95 °C (Holding Stage) and then 95 °C over 15 s followed by 1 min at 60 °C (40 cycles). Each primer set was assayed under these conditions in a 200–500 nM range to identify the concentration providing the highest sensitivity and specificity for each target sequence. No template controls were included for each primer pair and each qPCR reaction was carried out in duplicate. Gene-specific amplification was confirmed by a single peak in the melting-curve analysis. Calibration curves were built after the amplification of decreasing amounts of a cDNA pool, making dilutions at fivefold intervals to evaluate the qPCR efficiency for a given gene in each species. Standard curves displayed slope values between −3.6 and −3.1 and r^2 higher than 0.980 as it is recommended in the ABI Prism 7500 Real Time PCR System guidelines (Applied Biosystems SA). The amplification efficiency % for each primer pair in each species was calculated from the expression $[10^{(-1/s)} - 1] \times 100\%$, where S represents the slope of the plot of mean of cycles of quantification (Cq) against logarithm of the cDNA dilution. The efficiency range was between 1.91 and 2.06.

2.8.5. Statistical analysis of the data

The pharmacokinetic parameters and concentration data are reported as arithmetic mean ± SD. Parametric (ANOVA + Tukey) and non-parametric (Kruskal–Wallis) tests were used for the statistical comparison of the pharmacokinetic data obtained from the different experimental groups. Dose proportionality was indirectly determined by non-parametric analysis of the non-transformed dose-normalized IVM AUC_{0-LOQ} and Cmax, using the Kruskal–Wallis statistical test. The Cq values were converted into RQ via the delta-Cq method (Livak and Schmittgen, 2001), incorporating the calculated amplification efficiency for each primer pair. The RQ values were calculated using the average Cq of all samples as calibrator. The relative abundance of one specific mRNA (mean ± SEM) was normalized to the expression of reference genes and given as fold-change compared to control values. Statistical comparisons between the P-gp expression results in *H. contortus* obtained from untreated control and IVM-treated groups were made using the unpaired Student's *t*-test with the Welch correction if it was necessary. The statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, San Diego, CA, USA). In all cases, a value of $P < 0.05$ was considered statistically significant.

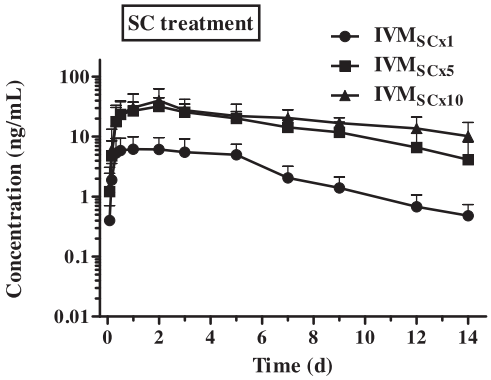

Fig. 1. Comparative mean (±SD) ivermectin (IVM) plasma concentration profiles ($n = 6$) of ivermectin (IVM), obtained after its administration by the subcutaneous (SC) route at either 0.2 (IVM_{SCx1}), 1.0 (IVM_{SCx5}) or 2.0 (IVM_{SCx10}) mg/kg to *Haemonchus contortus*-infected lambs.

3. Results

None of the animals involved in the current trial showed any adverse events such as diminishing food consumption and/or central nervous system toxicities (ataxia, postration, anorexia, etc.) after IVM treatments, even at the highest dose. The mean (±SD) IVM plasma concentrations profiles after its SC administration at different doses are shown in Fig. 1. In all experimental groups (SC treatments) IVM was quantified plasma from 1 h to 14 days post-treatment. Table 2 summarizes the IVM plasma pharmacokinetic parameters obtained after its SC administration at different doses to infected lambs. The AUC_{0-LOQ} represented 94.8 (IVM_{SCx1}), 90.2 (IVM_{SCx5}) and 75.8% (IVM_{SCx10}) of the $AUC_{0-\infty}$ for the three different experimental groups, confirming that the 14 days sampling time was an adequate period for estimation of plasma disposition kinetics of IVM after x1 and x5 doses, but fail to effectively represent the concentration vs time curve in the x10 treated group. The IVM AUC_{0-LOQ} significantly ($P < 0.05$) increased from 41.9 ± 20.1 ng.d/mL (IVM_{SCx1}) up to 221 ± 55.9 ng.d/mL (IVM_{SCx5}) and 287 ± 100.4 ng.d/mL (IVM_{SCx10}). The higher dose levels (x5 and x10) were also correlated with a significant enhancement of the IVM peak plasma concentrations, which were obtained at the same time post-treatments (Table 2). Furthermore, when the mean AUC_{0-LOQ} and Cmax values were normalized by the dose, differences among groups did not reach statistical significance ($P > 0.5$) showing a dose-proportional relationship. The MRT and *T½*el increased according to the dose

Table 2
Plasma pharmacokinetic parameters (mean ± SD) for ivermectin (IVM) obtained after the subcutaneous (SC) administration of IVM to lambs parasitized with *Haemonchus contortus* at different doses: 0.2 (IVM_{SCx1}), 1 (IVM_{SCx5}) and 2 (IVM_{SCx10}) mg/kg.

Pharmacokinetic parameters	SC treatment		
	IVM_{SCx1}	IVM_{SCx5}	IVM_{SCx10}
Cmax (ng/mL)	7.48 ± 3.44a	37.2 ± 11.6b	43.9 ± 21.2b
Tmax (d)	1.47 ± 1.21a	1.58 ± 0.66a	2.17 ± 2.40a
AUC_{0-LOQ} (ng.d/mL)	41.9 ± 20.1a	220.9 ± 55.9b	286.6 ± 100.4b
$AUC_{0-\infty}$ (ng.d/mL)	44.0 ± 20.5a	250.4 ± 82.6b	410.8 ± 212.0b
AUMC (ng.d^2/mL)	218.8 ± 102.3a	1764 ± 1226a,b	5146 ± 4819b
*T*½el (d)	2.94 ± 0.48a	3.81 ± 1.50a,b	6.73 ± 3.51b
*T*½abs (d)	0.45 ± 0.45a	0.69 ± 0.48a	0.80 ± 0.86a
MRT (d)	5.05 ± 0.96a	6.57 ± 2.37a,b	10.8 ± 5.06b
Normalized AUC*	41.9 ± 20.1a	44.2 ± 11.2a	28.7 ± 10.0a
Normalized Cmax*	7.48 ± 3.44a	7.43 ± 2.32a	4.40 ± 2.12a

Cmax: peak plasma concentration; **Tmax**: time to the Cmax; **AUC_{0-LOQ}**: area under the plasma concentration vs. time curve from 0 up to the quantification time; **$AUC_{0-\infty}$**: area under the concentration vs. time curve extrapolated to infinity; **AUMC**: area under the first moment curve; ***T*½el**: elimination half-life; ***T*½abs**: absorption half-life; **MRT**: mean residence time (obtained by non-compartmental analysis of the data).

* AUC_{0-LOQ} and Cmax values were dose-normalized dividing the observed value by 5 (IVM_{X5}) or 10 (IVM_{X10}). Pharmacokinetic parameters with different online letters are statistically different at $P < 0.05$.

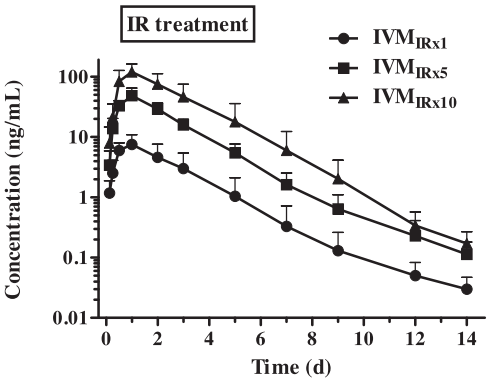

Fig. 2. Comparative mean (±SD) ivermectin (IVM) plasma concentration profiles ($n = 6$) of ivermectin (IVM), obtained after its administration by the intraruminal (IR) route at either 0.2 (IVM_{IRx1}), 1.0 (IVM_{IRx5}) or 2.0 (IVM_{IRx10}) mg/kg to *Haemonchus contortus*-infected lambs.

rate. This increment reached statistical significance only between IVM_{SCx1} and IVM_{SCx10} treatments.

The plasma concentrations of IVM obtained after its IR administration at different doses to lambs is shown in Fig. 2. IVM plasma concentrations were quantified between 3 h and 14 days post-administration in all experimental groups. Table 3 shows the plasma pharmacokinetics parameters obtained for IVM after its IR administration at different doses to parasitized lambs. IVM reached Cmax values of 8.67 ± 2.18 (IVM_{IRx1}), 48.3 ± 16.1 (IVM_{IRx5}) and 120 ± 42.5 ng/mL (IVM_{IRx10}) at similar times post-treatment, which were between 0.83 and 1 day post-treatment. The higher Cmax accounted for a significantly ($P < 0.05$) higher AUC value measured for the IVM_{IRx10} group (AUC = 323 ± 178 ng d/mL), compared to those obtained in the IVM_{IRx1} treatment (AUC = 20.8 ± 12.3 ng.d/mL). Similar to that observed after the SC treatment, when the AUC and Cmax values were normalized by the dose, no statistical ($P > 0.05$) differences in the absorption rate were observed among doses. While any effect on the *T*½el for IVM was observed between the highest and the lowest dose, similar MRT values were observed among groups.

The faecal egg counts (mean and range), adult *H. contortus* counts (mean and range) and resultant efficacy data obtained for the different experimental groups after IVM administration by the SC and IR routes, are shown in Tables 4 and 5, respectively. The low efficacy level (42–50%) obtained at the therapeutic dose (0.2 mg/kg) after both routes of administration confirms the high IVM-resistant status of the worms. The efficacy of IVM against adult *H. contortus* increased with the increment of the dose both, after the SC and IR treatment. After the SC treatment, an anthelmintic efficacy of 75% was observed for the administration of both, 5 and 10-times the therapeutic dose. The individual adult nematode counts in IVM_{SCx5} and IVM_{SCx10} resulted significantly ($P < 0.05$) lower to that observed in the IVM_{SCx1} and the untreated control Group. The efficacy estimated by FECRT after the SC administration of IVM reach 68, 91 and 92% after treatment at 0.2, 1 and 2 mg/kg, respectively. After the IR administration of IVM, the highest doses (x5 and x10) resulted in a significant ($P < 0.05$) reduction in adult *H. contortus* counts, compared to that observed both in the group treated at the therapeutic dose and the untreated control. A high efficacy was observed in both IVM_{IRx5} (96%) and IVM_{IRx10} (98%) groups. The FECRT after the IR administration of IVM reach 0, 87 and 94% after treatment at 0.2, 1 and 2 mg/kg, respectively.

Interestingly, after the SC administration of IVM at the three doses, the "true" efficacy, estimated by direct nematode counts, falls below the lower confidence limit calculated after the FECRT (Table 4). However, after its IR administration the "true" efficacy was found within the confidence interval, close to its upper limit (Table 5).

H. contortus P-gp expression was measured by a real time PCR approach. Different genes frequently used as references genes (RGs) in quantitative PCR (qPCR) experiments were currently screened. The actin (ACT) was selected as reference gene for qPCR normalization in *H. contortus*. Fig. 3 shows the P-gp homolog expression in the nematode parasite recovered from the different IVM-treated groups. The different IVM administration protocols

Table 3
Plasma pharmacokinetic parameters (mean ± SD) for ivermectin (IVM) obtained after the intraruminal (IR) administration of IVM to lambs parasitized with *Haemonchus contortus* at different doses: 0.2 (IVM_{IRx1}), 1 (IVM_{IRx5}) and 2 (IVM_{IRx10}) mg/kg.

Pharmacokinetic parameters	IR treatment		
	IVM_{IRx1}	IVM_{IRx5}	IVM_{IRx10}
Cmax (ng/mL)	8.67 ± 2.18a	48.3 ± 16.1a,b	119.7 ± 42.5b
Tmax (d)	0.83 ± 0.26a	1.00 ± 0.00a	1.00 ± 0.00a
AUC_{0-LOQ} (ng.d/mL)	20.8 ± 12.3a	120.9 ± 22.0a,b	323.3 ± 178.0b
$AUC_{0-\infty}$ (ng.d/mL)	20.8 ± 12.3a	121.1 ± 22.1a, b	323.6 ± 178.1b
AUMC (ng.d^2/mL)	50.3 ± 39.3a	276.7 ± 63.1a,b	787.7 ± 560.3b
*T½*el (d)	1.57 ± 0.13a	1.43 ± 0.17a,b	1.29 ± 0.10b
*T½*abs (d)	0.26 ± 0.12a	0.29 ± 0.05a	0.32 ± 0.15a
MRT (d)	2.20 ± 0.46a	2.30 ± 0.37a	2.28 ± 0.41a
Normalized AUC*	20.8 ± 12.3a	24.2 ± 4.40a	32.3 ± 17.8a
Normalized Cmax*	8.67 ± 2.18a	9.66 ± 3.21a	12.0 ± 4.25a

Cmax: peak plasma concentration; **Tmax**: time to the Cmax; **AUC_{0-LOQ}**: area under the plasma concentration vs. time curve from 0 up to the quantification time; **$AUC_{0-\infty}$**: area under the concentration vs. time curve extrapolated to infinity; **AUMC**: area under the first moment curve; ***T*½el**: elimination half-life; ***T*½abs**: absorption half-life; **MRT**: mean residence time (obtained by non-compartmental analysis of the data).

* AUC_{0-LOQ} and Cmax values were dose-normalized dividing the observed value by 5 (IVM_{x1}) or 10 (IVM_{x10}). Pharmacokinetic parameters with different online letters are statistically different at $P < 0.05$.

Table 4
Nematode egg counts[a] (range), reduction percentage of faecal egg counts (FECRT), adult *Heaemonchus contortus* counts and efficacy after the subcutaneous (SC) administration of ivermectin at 0.2 (IVM_{SCx1}) 1.0 (IVM_{SCx5}) and 2.0 (IVM_{SCx10}) mg/kg dose, in parasitized lambs.

Experimental group	Faecal egg counts[a] (range)				FECRT (%)[b] (LCL–UCL)		*H. contortus* counts[a] (range)		Efficacy[c] (%)
	Trial day −1		Trial day 14						
Control	8047	(4600–12,480)	1235	(750–2370)	–	–	2335a	(1930–2810)	–
IVM_{SCx1}	8267	(4720–12,240)	393	(110–650)	68	(44–82)	1353b	(850–1960)	42
IVM_{SCx5}	8120	(5680–13,400)	117	(60–300)	91	(79–96)	595c	(330–840)	75
IVM_{SCx10}	8377	(5880–13,360)	102	(60–220)	92	(84–96)	585c	(400–890)	75

[a] Arithmetic mean.
[b] FECRT estimated according to Coles et al. (1992).
[c] Efficacy estimated according Wood et al. (1995), using arithmetic means as suggested by McKenna (1997). UCL: upper confidence limit 95%; LCL: lower confidence limit 95%.
Adult *H. contortus* counts at day 14 post treatment with different online letters (a, b or c) are statistically different at $P < 0.05$.

used here (Experiment II) did not modify P-gp homolog expression in the recovered adult parasites compared to those recovered from the untreated control lambs.

4. Discussion

The effect of single SC or IR administration of IVM at three different doses (0.2, 1 and 2 mg/kg) on its plasma pharmacokinetic behaviour and efficacy against resistant-*H. contortus* in lambs was assessed. After SC IVM administration, the dose-related parameters AUC and Cmax increased with the dose increment. Compared to those obtained at the therapeutic dose, the AUC_{0-LOQ} and Cmax values after the x5 and x10 doses were significantly higher ($P < 0.05$). No differences ($P > 0.05$) in both AUC_{0-LOQ} and Cmax were observed between IVM_{SCx5} and IVM_{SCx10} treatments. The AUC_{0-LOQ} represented 80 and 70% of the $AUC_{0-\infty}$ in the IVM_{SCx5} and IVM_{SCx10} groups, respectively. This result demonstrates that the 0–14 days sampling period was not enough to accurately characterize the relative IVM plasma availability after its SC administration at 10 times the therapeutic dose. In fact, while a 30% increment was observed between the 1 and 2 mg/kg doses in the AUC_{0-LOQ} parameter, a 64% enhanced AUC was obtained when the estimation was extrapolated to infinity. The observed AUC and Cmax values for IVM after

Table 5
Nematode egg counts[a] (range), reduction percentage of faecal egg counts (FECRT), adult *Heaemonchus contortus* counts and efficacy after the intraruminal (IR) administration of ivermectin at 0.2 (IVM_{IRx1}), 1.0 (IVM_{IRx5}) and 2.0 (IVM_{IRx10}) mg/kg dose, in parasitized lambs.

Experimental group	Faecal egg counts[a] (range)				FECRT (%)[b] (LCL–UCL)		*H. contortus* counts[a] (range)		Efficacy[c] (%)
	Trial day −1		Trial day 14						
Control	5943	(1580–15,120)	4917	(2100–8260)	–	–	2425a	(1100–4370)	–
IVM_{IRx1}	5820	(2000–13,540)	6677	(1440–20,820)	0	(0–51)	1212a,b	(550–1700)	50
IVM_{IRx5}	6443	(1120–18,180)	637	(40–2300)	87	(56–96)	100b,c	(25–225)	96
IVM_{IRx10}	6010	(2120–12,980)	307	(140–780)	94	(86–97)	57c	(15–121)	98

[a] Arithmetic mean.
[b] FECRT estimated according to Coles et al. (1992).
[c] Efficacy estimated according Wood et al. (1995), using arithmetic means as suggested by McKenna (1997). UCL: upper confidence limit 95%; LCL: lower confidence limit 95%.
Adult *H. contortus* counts at day 14 post treatment with different online letters (a, b or c) are statistically different at $P < 0.05$.

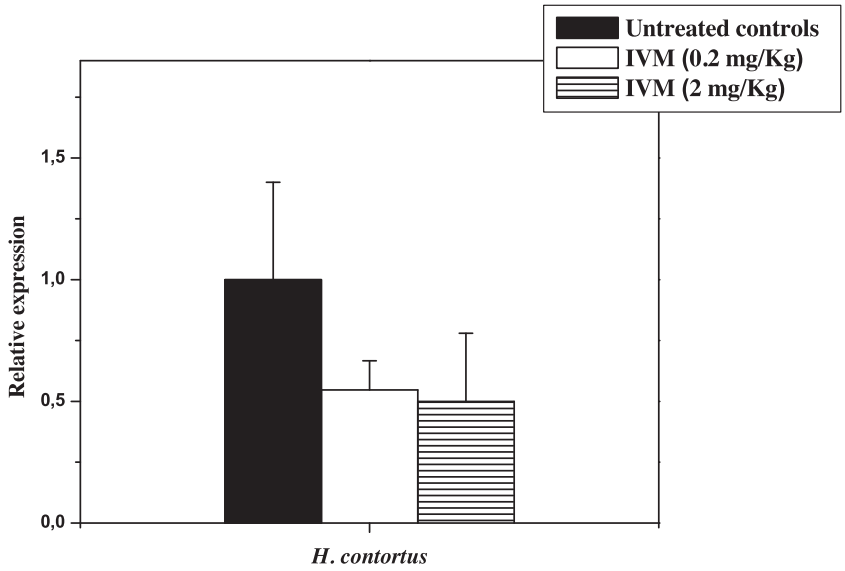

Fig. 3. Comparative P-glycoprotein mRNA expression profiles measured by real-time PCR (qPCR) in the liver, ileum mucosa and adult *Haemonchus contortus* specimens recovered from lambs treated with ivermectin (IVM) at 0.2 mg/kg and 2 mg/kg by the intraruminal (IR) route. Data are expressed as -fold change relative to control (vehicle) which was set to one, and are reported as the mean ± SEM ($n = 4$).

its SC administration at different doses exhibited no deviation from dose proportionality. Dose proportionality occurs when increases in the administered dose are followed by proportional enhancement in an exposure indicator such as AUC or Cmax. Since the dose-normalized AUC_{0-LOQ} and Cmax values for IVM after the three different doses were similar ($P > 0.05$) (Table 2), the IVM plasma drug exposure after its SC administration was proportional with the dose in the range of 1–10 times the therapeutic dose. The difference observed in the *T½*el between IVM_{SCx1} and IVM_{SCx10} (Table 2) was likely related to a slower IVM absorption rate after the highest dose treatment which may have limited the elimination process. This effect would not have relevant clinical consequences.

A similar result was observed after the IR administration of IVM at different doses. The observed AUC_{0-LOQ} and Cmax values after 0.2–2 mg/kg dose treatment exhibited no deviation from dose proportionality, with similar ($P > 0.05$) dose-normalized values (Table 3). After the IR administration, IVM AUC_{0-LOQ} represent 99.7 (IVM_{IRx1}), 99.8 (IVM_{IRx5}) and 99.9% (IVM_{IRx10}) of the total drug recovered from plasma ($AUC_{0-\infty}$) in the different groups, showing that the sampling time design was adequate. There were no significant differences in other pharmacokinetic parameters such as *T½*abs and MRT after the administration of different IVM doses (Table 3).

The *H. contortus* isolate used in the current experiment was highly resistant to IVM. A low efficacy pattern (42–50%) was observed after both the SC (Table 4) and IR (Table 5) IVM treatments at the therapeutic dose. Traditionally, a similar efficacy against susceptible *H. contortus* isolates could be obtained after the administration of IVM by the oral or SC routes (Borgsteede, 1993). However, an improved efficacy of different macrocyclic lactone compounds (moxidectin, abamectin, IVM) against resistant *Trichostrongylus colubriformis* or *H. contortus* has been reported after the oral/IR compared to the SC treatment (Gopal et al., 2001; Alka et al., 2004; Lloberas et al., 2012). This route of administration-related efficacy pattern was only observed after the highest dose treatment (x5 and x10), in which the IR treatment resulted in a significantly higher efficacy compared to the SC administration. Higher IVM concentration in the abomasal content was observed after its IR administration compared to that observed after the SC treatment (Lloberas et al., 2012). Since drug accumulation in gastrointestinal nematodes appears to be mainly related to drug diffusion from the surrounding medium (gastrointestinal fluids), the enhanced IVM concentration in abomasal content after the IR administration may have accounted for an increased drug accumulation in *H. contortus*, explaining the improved efficacy observed after the IR treatment at the highest doses. In fact, Lloberas et al. (2012) quantified a greater amount of IVM in *H. contortus* recovered from lambs treated by the IR route compared to those recovered from lambs treated SC. These enhanced IVM concentrations explain the higher efficacy observed after the IR treatment.

After both SC and the IR treatments, the higher the dose used the greater the anthelmintic efficacy against resistant-*H. contortus*. After the SC treatment, efficacy increased from 42% (therapeutic dose) to 75% (dose x5 and x10). The lack of differences between x5 and x10 doses could be given by a similar plasma drug exposure and, thus, an equivalent parasite exposure. However, it is likely that an improved efficacy may have been observed at a period of time longer than 14 days post-treatment. As previously mentioned, after the highest dose treatment a marked increment in the $AUC_{0-\infty}$ was observed, compared to that observed for the AUC_{0-LOQ}. Consequently, parasite drug exposure beyond 14 days will be likely higher for the x10 compared to the x5 treated group and an enhanced clinical efficacy may be obtained.

The results reported here demonstrate that, at least with the *H. contortus* isolate under investigation, individual parasites do not respond uniformly to treatment. Some were killed by a therapeutic IVM dose, other tolerated this dose but were eliminated by treatment at the highest doses (therapeutic dose x5 or x10), and some particular individuals survived even 10 times the therapeutic dose. This "dose-related behaviour" may be explained by two main factors. First and likely the most important one, is the "genetic factor", which is due to genetic diversity in the parasite population (Prichard, 2001). High genetic diversity has been described for different *H. contortus* populations such as genes encoding β-tubulins (Kwa et al., 1994; Beech et al., 1994), P-gp (Blackhall et al., 1998a; Sangster et al., 1999) and glutamate-gated chloride channel (GluClR) subunits (Blackhall et al., 1998b). Macrocyclic lactones resistance is quite complex, with mechanisms varying both within and between species (Gill and Lacey, 1998). The development of resistance to IVM requires of the simultaneous mutation of several genes to develop a high level of resistance (Martin et al., 2002). For example, a combination of two of the genes (*avr-14* and *avr-15*) produced a 13-fold increase in resistance in the model nematode *C. elegans* (Dent et al., 2000). Furthermore, changes on GABA receptors (Feng et al., 2002) and overexpression of membrane drug transporters including P-gp (Williamson et al., 2011; Dicker et al., 2011; Janssen et al., 2013; De Graef et al., 2013) have been associated with IVM resistance in different helminth parasites. In this context, the variation in response according to the administered dose level observed in the current trial may be explained by a genetic diversity within the isolate.

A second factor which may help to explain the differences in drug susceptibility of the *H. contortus* isolate, is related to drug diffusion and accumulation into the worm ("pharmacokinetic factor"). Pharmacokinetics is defined as the study of the drug absorption, distribution, metabolism, and excretion, and predicts the time course of drug throughout the body (Gibaldi and Perrier, 1982). The most commonly employed approach to the pharmacokinetic characterization of a drug is to represent a body as a system of compartments (compartment models), even though these compartments usually have no physiologic or anatomic reality. The physiological pharmacokinetic models incorporate physiological, anatomical and physicochemical data, and provide an exact description of the time course of drug concentration in any organ or tissue (Gibaldi and Perrier, 1982). However, the kinetic of a drug in a particular organ or tissue may be far from homogeneous. Different experimental trials support this assertion. Alvarez et al. (2011) investigated the accumulation (drug exposure) of albendazole and its active metabolite, albendazole sulphoxide, in resistant *H. contortus* recovered from two different locations within the abomasum (worms attached to the abomasal mucosa or "free" in the luminal abomasal content). Significantly higher ($P < 0.05$) drug accumulation was observed in *H. contortus* recovered from the abomasal fluid content, compared to those measured in resistant worms attached to the abomasal mucosa. A favourable partitioning process between fluid/worm compared to that obtained for the mucosa/worm, may have facilitated the greater drug accumulation observed in unattached worms collected from the abomasal fluid. The observed differential pattern of drug exposure according to worm location within the abomasum may have marked influence on the survival of resistant parasites after an anthelmintic treatment. Furthermore, the differential efficacy of IVM against *Oesophagostomum dentatum* (96.1–96.9%) and *O. quadrispinulatum* (53.2%), has been associated to differences on the host-parasite relationship (*O. dentatum* is predominantly localized in the distal part of large intestine and *O. quadrispinulatum* is confined to the proximal part) and on the pharmacokinetic disposition of IVM in the large intestine (Várady et al., 1996). In conclusion, both the genetic variability and the potential differences on drug accumulation according the *H. contortus* location within the abomasum, may have accounted for the observed differences in efficacy related to the IVM dose level.

The intensive use of macrocyclic lactones during the last 30 years to control parasitic diseases in ruminant species has led to the emergence of high levels of resistance, mainly in nematodes of sheep and goats but also in cattle gastrointestinal parasites (Kaplan, 2004; Demeler et al., 2009). Significantly different affinities by mammalian P-gp have been reported among macrocyclic lactones using in vitro assessments (Lespine et al., 2007). Ménez et al. (2012) reported that IVM increased Mdr1 levels in JWZ murine hepatic cells by reducing the degradation rate of the mRNA. In addition, the P-gp (P-gp-A)-mediated drug efflux was proposed among the mechanisms of nematode resistance to the macrocyclic lactones (Xu et al., 1998; Prichard and Roulet, 2007). P-gp homologues are thought to play an important role in the resistance of nematodes against macrocyclic lactones, and available information suggests that induction of P-gp expression in response to IVM treatment may also exist in nematodes (Ménez et al., 2012). Up-regulation of these proteins would serve to eliminate the drug from the parasite, limiting the drug accumulation. Moreover, modifications on the pattern of nematode P-gp expression have been observed in resistant nematodes recovered from macrocyclic lactone-treated lambs at 0.5 and 1 days post-treatment compared to those parasites recovered from untreated control animals (Prichard and Roulet, 2007; Lloberas et al., 2013). Considering these observations, the gene expression profiles of P-pg in *H. contortus* were analysed by quantitative PCR in the current experimental trial. In order to minimize the use of experimental animals, the molecular studies were carried out in accordance to the protocol designed for the current efficacy studies. The IR administration of the highest doses (x5 and x10) of IVM did not cause any significant change on P-gp mRNA levels in the adult nematodes parasites recovered from treated animals at 14 days post-treatment. According to data reported by Ménez et al. (2012), the induction of Mdr1 in mouse hepatocyte cultured cells in the presence of IVM treatment is transient, since the rapid increase in Mdr1 mRNA levels reached a plateau at 24 h or 48 h and then decreased after longer exposure times (72 h). More recently, Albérich et al. (2014) showed that a single therapeutic dose administration of IVM in mice did not change the expression of any detoxification genes

evaluated, included Mdr1. Taking into account this information, we conclude that a single dose of IVM does not increase the P-gp mRNA levels in *H. contortus* and/or 14 days post-treatment sampling time may be too late to detect P-gp mRNA potential changes mediated by the IVM treatment.

The observed results indicate that enhanced IVM systemic exposure associated with the highest doses, resulted in significantly increased drug efficacy. Macrocyclic lactones are reversibly exchanged between the bloodstream and the gastrointestinal tract (Lifschitz et al., 2000), the enhanced drug concentrations associated to the increasing administered doses, may account for gastrointestinal nematodes being exposed to toxic drug concentrations for extended periods of time. This finding helps to explain the "reversion" of the drug resistance phenomenon observed in the current trial after administration of IVM at higher dose levels by the IR route. The large IVM concentrations achieved in the abomasal content after its IR administration compared to the SC injection (Lloberas et al., 2012), may have accounted for a greater amount of drug reaching the adult parasite. An enhanced abomasal drug exposure may explain the improved efficacy against this recalcitrant *H. contortus* strain observed only after the IR administration at 5- and 10-fold the IVM therapeutic dosage.

The work reported here contributes to the understanding of the pharmacokinetic impact of using high IVM doses to control resistant helminth parasites. As it was shown here, the IVM resistance may be overcome by increasing the amount of the active drug at the biophase. In fact, under our experimental conditions an IR IVM dose as high as 5- to 10-fold the therapeutic dosage was necessary to reach an acceptable efficacy level against resistant *H. contortus*. The inconvenience of recommending high dose rates may be associated with the selection of highly resistant nematodes, in addition to the impact of drug residues, withdrawal times, etc., which surely may preclude its use as a "practical" strategy when resistant parasites are present. An integrated pharmacokinetic-drug effect contribution to the issue is the main outcome of the work described here.

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