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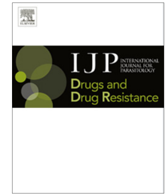

Comparative tissue pharmacokinetics and efficacy of moxidectin, abamectin and ivermectin in lambs infected with resistant nematodes: Impact of drug treatments on parasite P-glycoprotein expression ☆

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ABSTRACT

The high level of resistance to the macrocyclic lactones has encouraged the search for strategies to optimize their potential as antiparasitic agents. There is a need for pharmaco-parasitological studies addressing the kinetic-dynamic differences between various macrocyclic lactones under standardized *in vivo* conditions. The current work evaluated the relationship among systemic drug exposure, target tissue availabilities and the pattern of drug accumulation within resistant *Haemonchus contortus* for moxidectin, abamectin and ivermectin. Drug concentrations in plasma, target tissues and parasites were measured by high performance liquid chromatography. Additionally, the efficacy of the three molecules was evaluated in lambs infected with resistant nematodes by classical parasitological methods. Furthermore, the comparative determination of the level of expression of P-glycoprotein (P-gp2) in *H. contortus* recovered from lambs treated with each drug was performed by real time PCR. A longer persistence of moxidectin ($P < 0.05$) concentrations in plasma was observed. The concentrations of the three compounds in the mucosal tissue and digestive contents were significant higher than those measured in plasma. Drug concentrations were in a range between 452 ng/g (0.5 day post-treatment) and 32 ng/g (2 days post-treatment) in the gastrointestinal (GI) contents (abomasal and intestinal). Concentrations of the three compounds in *H. contortus* were in a similar range to those observed in the abomasal contents (positive correlation $P = 0.0002$). Lower moxidectin concentrations were recovered within adult *H. contortus* compared to abamectin and ivermectin at day 2 post-treatment. However, the efficacy against *H. contortus* was 20.1% (ivermectin), 39.7% (abamectin) and 89.6% (moxidectin). Only the ivermectin treatment induced an enhancement on the expression of P-gp2 in the recovered adult *H. contortus*, reaching higher values at 12 and 24 h post-administration compared to control (untreated) worms. This comparative pharmacological evaluation of three of the most used macrocyclic lactones compounds provides new insights into the action of these drugs.

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1. Introduction

Among the available chemical groups used to control the parasitic diseases in ruminants, the macrocyclic lactones (MLs) have been the most used drugs during the last 30 years. The intensive use of these broad-spectrum antiparasitic compounds has led to the emergence of high levels of resistance mainly in nematodes of sheep and goats but also in cattle gastrointestinal (GI) parasites (Kaplan, 2004; Demeler et al., 2009). This high level of resistance to the MLs has encouraged the search for strategies to optimize their enormous potential as antiparasitic agents in an attempt to extend their life span, particularly in geographic areas where resistance is not yet fully present.

The use of the MLs by the oral route in sheep and goats has led to some advantageous efficacy patterns against resistant nematodes in comparison to subcutaneous treatment (Gopal et al., 2001; Lloberas et al., 2012). Moreover, different efficacy performances have been described for ivermectin (IVM), abamectin (ABM) and moxidectin (MXD) in sheep infected with resistant GI nematodes (Barnes et al., 2001; Vickers et al., 2001; Alka et al., 2004). Although these ML compounds belong to two different chemical families,

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avermectins (IVM, ABM) and mylbemycins (MXD), they share a common ML structure and many physicochemical and pharmacological properties. In spite of the fact that compounds in both families seem to exert their antiparasitic effect by the same mode of action, some clear pharmacokinetic (Lanusse et al., 1997; Molento et al., 2004) and also pharmacodynamic differences between IVM and MXD (Njue et al., 2004) have been described and it may account for their differential effectiveness against certain resistant nematodes, which has been recently reviewed by Prichard et al. (2012). However, there is still a need for pharmaco-parasitological studies addressing these kinetic-dynamic differences under standardized *in vivo* conditions.

The P-glycoprotein (P-gp)-mediated drug efflux was proposed among the mechanisms of nematode resistance to the MLs (Xu et al., 1998; Prichard and Roulet, 2007). Significantly different affinities by mammalian P-gp have been reported among MLs using *in vitro* assessments (Lespine et al., 2007). Additionally, modifications on the pattern of nematode P-gp expression have been observed in resistant nematodes recovered from animals treated with MLs (Prichard and Roulet, 2007). To gain some further insight into the comparative pharmacology of the therapeutically-used ML compounds, the work reported here was designed to establish the relationship among systemic drug exposure, target tissue availabilities and the pattern of drug accumulation within resistant *Haemonchus contortus* for three of the most used compounds. The comparative assessment of the systemic and tissue pharmacokinetics of MXD, ABM and IVM was complemented with the simultaneous evaluation of the clinical efficacy of the three molecules in lambs infected with resistant nematodes. Furthermore, the work included the comparative determination of the level of expression of the *H. contortus* drug transporter, P-gp 2, in adult worms recovered from lambs treated with each of the MLs.

2. Material and methods

2.1. Animals

Sixty-four (64) Romney Marsh lambs (27.2 ± 4.48 kg), naturally infected with resistant GI nematodes were involved in this trial. The isolate was from a sheep Experimental Unit (Reserva 8, Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina) with a parasite control program 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, personal communication). The reduction of the faecal egg counts obtained after treatment with IVM the years prior to performing the trial described here was below 80% (Entrocasso et al., 2008; Lifschitz et al., 2010a). The selection of the animals was based on worm egg per gram counts (epg). On day-1 all lambs were checked for epg, ear tagged and the individual body weights were recorded. Experimental animals had an average of 4071 ± 1630 epg counts ranging from 2600 to 8200. Animals were allocated in a paddock and fed on a lucerne/white and red clover pasture during the experiment and for 20 days before the start of the clinical efficacy study. 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, treatments and samplings

Experimental lambs were assigned into four (4) experimental groups. Group A (n = 10) remained as untreated controls. Animals in group B–D (n = 18 in each group) received MXD (Cydectin®, Fort Dodge, Argentina), ABM (Abamin®, Rosenbusch, Argentina) or IVM (Ivomec® Oral, Merial, Uruguay) intraruminally at 0.2 mg/kg, respectively. The intraruminal (IR) route was selected instead of oral administration in order to avoid the closure of the esophageal groove and hence, to minimize the variability in drug uptake. To study the distribution of the three drugs to target tissues and parasites and to measure the nematode's P-gp2 expression in *H. contortus*, four animals from groups B–D were sacrificed at 0.5, 1 and 2 days post-administration and samples of blood, abomasal and small intestine (craneal jejunum) contents and mucosal tissue were taken following the procedures described in Lifschitz et al. (2000). From the abomasum of each animal, 20 adult females of *H. contortus* were rapidly recovered, gently washed in saline solution at 4 °C and immediately frozen in vials in liquid nitrogen to study the nematode P-gp2 expression. Then, the total mass of *H. contortus* was recovered from the abomasal contents, gently washed in saline solution at 4 °C, blotted on coarse filter paper and immediately frozen (−20 °C) in vials to measure the drug concentration in the parasites. Additionally, four animals of group A (untreated group infected with a resistant *H. contortus* isolate) and four lambs artificially infected (7000 L_3 each lamb) with a susceptible strain of *H. contortus*, were sacrificed and the adult parasite females were collected as a control to measure the nematode P-gp2 expression.

To characterize the efficacy of MXD, ABM and IVM, faecal samples were collected from the lambs in each experimental group at days-1 and 14 post-treatment in order to estimate the epg count reduction (Coles et al., 1992). For the plasma disposition study (n = 6 in each group), jugular blood samples (5 ml) were collected into heparinized vacutainer® tubes (Becton Dickinson, USA) prior to and at 0, 3, 6, 9 h and 1, 2, 3, 4, 6, 8, 10 and 13 days post-treatment. Blood samples were centrifuged at 2000*g* for 20 min and the recovered plasma was kept in labeled vials. Plasma, tissues and GI contents samples were stored at −20 °C until analyzed by high performance liquid chromatography (HPLC). Additionally, at 14 days post-treatment, all the animals involved in the pharmacokinetic study and six animals from the untreated control group were sacrificed by captive bolt gun and rapidly exsanguinated. Abomasum and different gut sections were identified and isolated (small and large intestine) and the contents analyzed to record the different parasite stages following the World Association for the Advancement of Veterinary Parasitology guidelines (Wood et al., 1995).

3. Analytical procedures

3.1. Pharmacological determinations

3.1.1. MLs chemical extraction and derivatization

The extraction of each ML from spiked and experimental plasma samples was carried out following the technique first described by Alvinerie et al. (1993), slightly modified by Lifschitz et al. (1999, 2000). Basically, 1 ml aliquot of plasma, 0.5 g of GI samples (mucosas and contents) or 100 mg of parasites were combined with the internal standard compound (doramectin) and then mixed with 1 ml of acetonitrile–water (4:1). The preparation was mixed (Multi Tube Vortexer, VWR Scientific Products, West Chester, PA, USA) over 15 min. Parasites and GI tissue/content samples were sonicated in an ultrasonic bath for 10 min. (Transsonic 570/H, Laboratory Line Instruments Inc., Melrose Park, IL, USA). The supernatant was manually transferred into a tube and the procedure was repeated once more for the GI tissue/content and parasite samples. The supernatant was then placed on the appropriate rack of an Aspec XL sample processor (Gilson, Villiers Le Bel, France) to perform the solid-phase extraction (Lifschitz et al., 1999). The derivatization of MLs was done with 100 μl of a solution of

N-methylimidazole (Sigma Chemical, St. Louis, MO, USA) in acetonitrile (1:1) and 150 μl of trifluoroacetic anhydride (Sigma Chemical, St. Louis, MO, USA) solution in acetonitrile (1:2) (De Montigny et al., 1990). After completion of the reaction (<30 s), an aliquot (100 μl) of this solution was injected directly into the HPLC system.

3.1.2. Chromatographic conditions

MLs 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 (Kromasil, Eka Chemicals, Bohus, Sweden, 5 μm, 4.6 mm × 250 mm) and an acetic acid 0.2% in water/methanol/acetonitrile (1.6/60/38.4) mobile phase at a flow rate of 1.5 ml/min at 30 °C (Lifschitz et al., 1999). MLs were detected with a fluorescence detector (Shimadzu, RF-10 Spectrofluorometric detector, Kyoto, Japan), reading at 365 nm (excitation) and 475 nm (emission wavelength). A validation of the analytical procedures used for extraction and quantification of each ML from plasma, GI mucosa and contents and parasites were performed before starting analysis of the experimental samples obtained during the pharmacokinetic trial. Calibration curves were established using least squares linear regression analysis, and correlation coefficients (r) and coefficient of variations (CV) were calculated.

3.2. Parasitological techniques

Individual faecal egg counts were performed using the modified McMaster technique (Roberts and O'Sullivan, 1949). The anthelmintic efficacy of the treatments was evaluated by the faecal egg count reduction test (FECRT), calculated according to the formula:

$$\mathrm{FECRT}\,(\%) = 100 \times (1 - T/C),$$

where T is the arithmetic mean epg counts in the treated group at 14 days post treatment and C is the arithmetic mean epg counts in the untreated control group at 14 days post treatment (Coles et al., 1992). The 95% confidence intervals were calculated as reported by Coles et al. (1992). Direct adult nematode counts of animals from experimental groups were determined 14 days after treatment 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 a given parasite species (S) in a single treatment group (T) when compared with an untreated control (C):

$$\%E = [(\text{Mean of } S \text{ in } C - \text{Mean of } S \text{ in } T)/\text{Mean of } S \text{ in } C] \times 100$$

The geometric mean was used according to the recommendations of Wood et al. (1995).

3.3. Quantification of P-gp expression in H. contortus

3.3.1. Isolation of total RNA and reverse transcription

Total RNA was isolated from about 30 mg of frozen resistant *H. contortus* recovered from control and treated lambs. Susceptible adults *H. contortus* recovered from untreated lambs were also processed as a positive control. The total RNA was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen). Briefly, 1 ml of Trizol was added to an aliquot (30 mg) of parasites and homogenized manually several times using a 1 ml syringe. Then, samples were purified with a phenol–chloroform extraction process. Total RNA purity and concentration were determined by measuring the absorbance at 260 and 280 nm after dilution of the sample 1:500 in RNase-free water. In all samples the 260/280 ratio was ⩾1.8. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's procedure. Complementary DNA was stored at −20 °C until Real-time PCR analysis.

Table 1
GenBank accession numbers, sequences of primer sets used for real-time PCR and the respective product sizes.

Gene name	GenBank accession number	5′–3′ Primer sequence	Product size (bp)
Actin	DQ080917.1	f: gctcccagcacgatgaaaa r: accaatccagacagagtatttg	66
P-gp2	AF003908.1	f: cggcagcagatctcatggt r: tcggttagacgagctgtgagatt	61

P-gp: P-glycoprotein; f: forward; r: reverse.

3.3.2. Real-time quantitative PCR

H. contortus mRNA sequences of target and reference genes were obtained from the GenBank web site (http://www.ncbi.nlm.nih.gov). Primer sequences used for nematode P-gp2 expression and quantification analysis were designed using Primer Express™ Software 2.0 (Applied Biosystems) based on the sequences published by Xu et al. (1998) for P-gp2 and subjected to primer test analyses to exclude dimer formation (Primer Test Document application in Primer Express™ Software). The set of primers for the amplification of *H. contortus* actin mRNA was obtained from the sequences published by Kotze and Bagnall (2006). Primer sequences, GenBank accession numbers and product sizes used for Real-time PCR analysis are summarized in Table 1. Each primers set was optimized in a 200–500 nM range to identify the concentration that provided the highest sensitivity. Real-time quantification was carried out in an ABI Prism 7500 Real Time PCR System (Applied Biosystems). The reaction mixture included 10 μl of PCR SYBR Green Master Mix 2× (Applied Biosystems), 2 μl of each primer set (200 nM), 1 μl of cDNA diluted 1:250 and 7 μl of water to obtain a final volume of 20 μl. Amplification was carried out in a 96-wells plate. The Real-time PCR was run under the following thermal profile: 50 °C for 2 min. and 95 °C for 10 min. (holding stage), 40 cycles of 95 °C for 15 s and 60 °C for 1 min. (cycling stage). Validation curves were performed with decreasing amounts of a cDNA pool diluted at 5-fold intervals to evaluate the Real-time PCR efficiency. Standard curves with $-3.6 < \text{slope} < -3.1$ and precision (r^2) higher than 0.985 were considered as acceptable as it is recommended in the ABI Prism 7500 Real Time PCR System guidelines (Applied Biosystems S.A.). To measure the nematode P-gp2 expression, the mRNA relative quantification was carried out using the $\Delta\Delta Ct$ method (Livak and Schmittgen, 2001). The slope curves were −3.56 (P-gp2 mRNA) and −3.51 (actin mRNA). The r^2 values were 0.998 for both genes under study.

4. Pharmacokinetic and statistical analysis of the data

The plasma concentrations versus time curves obtained after each treatment in each individual animal were fitted with the PK Solutions 2.0 (Ashland, Ohio, USA) computer software. Pharmacokinetic parameters were determined using a non-compartmental model method. The peak concentration (C_{max}) was read from the plotted concentration–time curve in each individual animal. The area under the concentration vs. time curves (AUC) were calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity by dividing the last experimental

concentration by the terminal slope (λz). The elimination half-life (t ½ el) was calculated as $\ln 2/\lambda z$. ML plasma concentrations and all the estimated pharmacokinetic parameters are reported as mean ± SD. Statistical moment theory was applied to calculate the mean residence time (MRT) as follows:

$$\mathrm{MRT} = \mathrm{AUMC}/\mathrm{AUC}$$

where AUC is as defined previously, and AUMC is the area under the curve of the product of time and drug concentration vs. time from zero to infinity (Gibaldi and Perrier, 1982).

Faecal egg and nematode counts (reported as arithmetic mean ± SD) were compared by non-parametric ANOVA (Kruskal–Wallis test). Mean pharmacokinetic parameters for each compound were statistically compared using ANOVA. The assumption that the data obtained after treatments have the same variance was assessed. A non-parametric ANOVA (Kruskal–Wallis test) was used where significant differences among standard deviations were observed. The statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, CA, USA). A value of $P < 0.05$ was considered statistically significant.

5. Results

The comparative plasma disposition kinetics of the MLs showed some differences among the drugs under study. Slightly higher plasma concentration profiles were obtained after the treatment with ABM compared to those of MXD and IVM. Although the systemic drug exposure (measured as AUC) was similar ($P > 0.05$) among the three compounds, the C_{max} of ABM (13.6 ng/ml) was significantly higher than that measured after the IVM treatment (9.00 ng/ml). Whereas MXD was detected in plasma until day 13 post-treatment, ABM and IVM were measured in the all the experimental animals only up to 8 days post-treatment. The longer persistence of MXD plasma concentrations accounted for a significant longer t ½ el and MRT compared to ABM and IVM. The ML plasma concentration profiles obtained after the IR administration to lambs are compared in Fig. 1. The main pharmacokinetic parameters for the three MLs under study are shown in Table 2.

High drug concentrations were recovered in the GI target tissues. The concentrations of the three compounds in the mucosal tissue and digestive contents were significant higher than those measured in plasma. Drug concentrations were in a range between 452 ng/g (0.5 day post-treatment) and 32 ng/g (2 days post-treatment) in the GI contents (abomasal and intestinal) and between 231 ng/g (0.5 day post-treatment) and 30 ng/g (2 days post-treatment) in the mucosal tissue. Whereas the concentrations in GI contents and mucosal tissues were similar for the three MLs at 0.5 and 1 day post-treatment, MXD concentrations were significantly lower compared to IVM and ABM at 2 days post-treatment. Mean ML concentrations in GI contents and mucosal tissues are compared in Fig. 2 and Table 3.

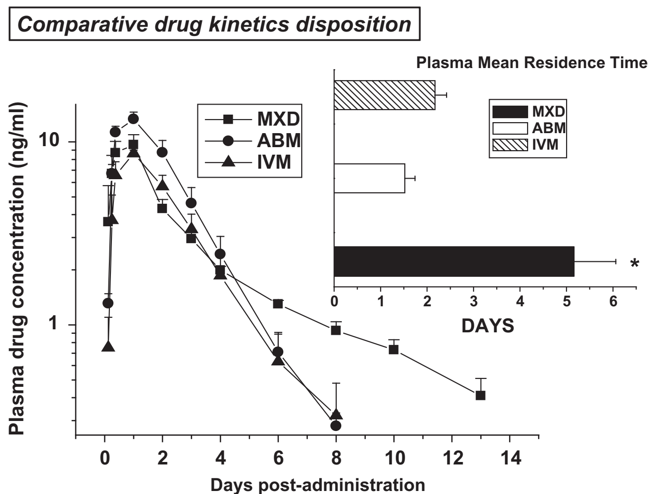

Fig. 1. Mean (±SD) ($n = 6$) moxidectin, (MXD), abamectin (ABM) and ivermectin (IVM) plasma concentrations obtained after their intraruminal (IR) administration (0.2 mg/kg) to nematode infected lambs. The insert shows the comparative plasma mean residence time obtained for the three compounds under study. (*) Values for MXD are statistically different from those obtained after the administration of ABM and IVM at $P < 0.05$.

Table 2
Mean (±SD) plasma pharmacokinetic parameters for moxidectin, (MXD), abamectin (ABM) and ivermectin (IVM) ($n = 6$) obtained after their intraruminal (IR) administration (0.2 mg/kg) to lambs.

Plasma kinetic parameters	MXD	ABM	IVM
T_{max} (days)	0.54 ± 0.37^{a}	0.90 ± 0.26^{a}	1.06 ± 0.52^{a}
C_{max} (ng/ml)	11.3 ± 2.87^{ab}	13.6 ± 2.64^{a}	9.02 ± 1.47^{b}
AUC (ng d/ml)	29.5 ± 3.87^{a}	34.8 ± 11.3^{a}	23.6 ± 7.63^{a}
t 1/2 el (days)	4.70 ± 2.17^{b}	1.10 ± 0.25^{a}	1.15 ± 0.30^{a}

T_{max}: time to peak plasma concentration. C_{max}: peak plasma concentration. AUC: area under the concentration vs time curve extrapolated to infinity. t ½ el: elimination half-life. Values with different superscript among MLs are statistically different at $P < 0.05$.

The *in vivo* drug concentrations in *H. contortus* were characterized at different times post-treatment. Concentrations of the ML measured in *H. contortus* were in a similar range to those observed in the abomasal content (positive correlation $P = 0.0002$). Significant differences among the MLs were observed at day 2 post-treatment, with lower MXD concentrations recovered within adult *H. contortus* compared to ABM and IVM (Fig. 3). Despite the lower concentrations recovered in the abomasal contents and accumulated within the parasite, the total mass of *H. contortus* specimens recovered were lower ($P < 0.05$) after the administration of MXD at day 1 and 2 post-treatment (0.13–0.14 g) compared to ABM (1.13–1.50 g) and IVM (1.02–1.38 g).

The faecal egg counts obtained for all the experimental groups, including the results of the FECRT and upper and lower confidence limits (95%) are shown in Table 4. The low percentage of reduction in the eggs counts in faeces indicates the presence of GI nematodes resistant to the ML compounds. The FECRT was 0% for IVM as well as for ABM and reached 86% after the MXD treatment. The efficacy against *H. contortus* showed significant differences among the MLs. The efficacy was 20.1% (IVM), 39.7% (ABM) and 89.6% (MXD). Efficacies against other abomasal, small and large intestine nematode species were above 98% after the treatment with each ML. The adult nematode counts and resultant clinical efficacy obtained for the MXD, ABM and IVM treatments are shown in Table 5.

The pattern of the drug-transporter P-gp mRNA expression in *H. contortus* exposed to the three compounds was established. First of all, a comparison of the level of P-gp2 expression in susceptible and resistant adults *H. contortus* recovered from untreated lambs was performed. The relative quantification showed that the expression of this gene was significantly higher ($P < 0.05$) in resistant (1.00 ± 0.42) than in susceptible (0.32 ± 0.14) *H. contortus* adult specimens. Interesting data emerged from the comparison of P-gp2 expression in the resistant adult *H. contortus* collected from untreated lambs and those recovered from ML-treated lambs. The IVM treatment induced an enhancement on the expression of P-gp2 in the recovered adult *H. contortus* reaching significantly higher values at 12 and 24 h post-administration. Although the P-gp2 expression tended to increase at 12 h after the MXD treatment, there was not a significant change on the transporter expression in the resistant *H. contortus*. On the other hand, P-gp2 expression in the *H. contortus* recovered after the ABM treatment

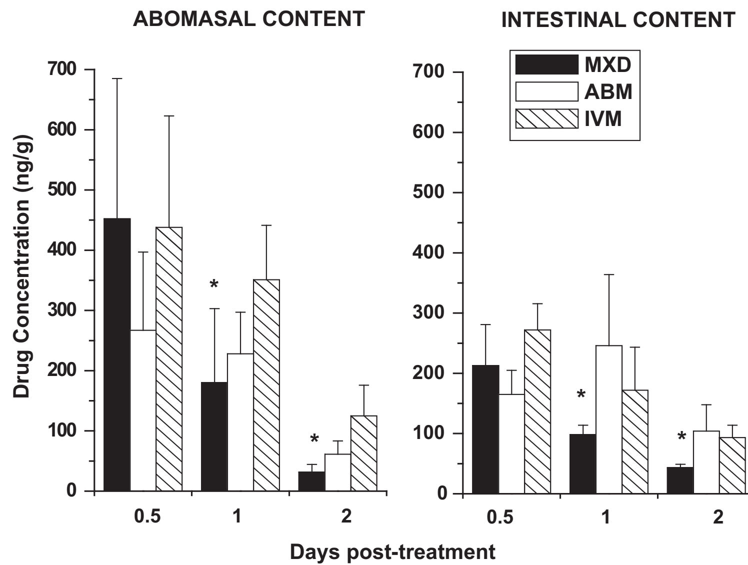

Fig. 2. Comparative mean (±SD) ($n = 4$) moxidectin, (MXD), abamectin (ABM) and ivermectin (IVM) concentrations in abomasal and intestinal contents measured after their intraruminal (IR) administration (0.2 mg/kg) to nematode infected lambs. (*) Values for MXD are statistically different from those obtained after the administration of ABM and IVM at $P < 0.05$.

Table 3
Comparative moxidectin (MXD), abamectin (ABM) and ivermectin (IVM) concentrations measured in abomasal and intestinal mucosal tissues after their intraruminal administration (0.2 mg/kg) to nematode infected lambs.

Drug concentration (ng/g)						
Time post-treatment (days)	Abomasal mucosa			Intestinal mucosa		
	MXD	ABM	IVM	MXD	ABM	IVM
0.5	65.5 ± 9.74[a]	76.8 ± 11.6[a]	76.5 ± 9.83[a]	231 ± 83[a]	203 ± 11.5[a]	198 ± 55.7[a]
1	48.3 ± 12.7[a]	78 ± 24.2[a]	58.2 ± 8.75[a]	134 ± 41.7[a]	152 ± 12.4[a]	117 ± 26.8[a]
2	30.1 ± 5.80[b]	64.6 ± 45.4[ab]	42.6 ± 4.53[a]	57.1 ± 5.30[b]	118 ± 85.9[a]	128 ± 51.4[a]

Values with different superscript among MLs are statistically different at $P < 0.05$.

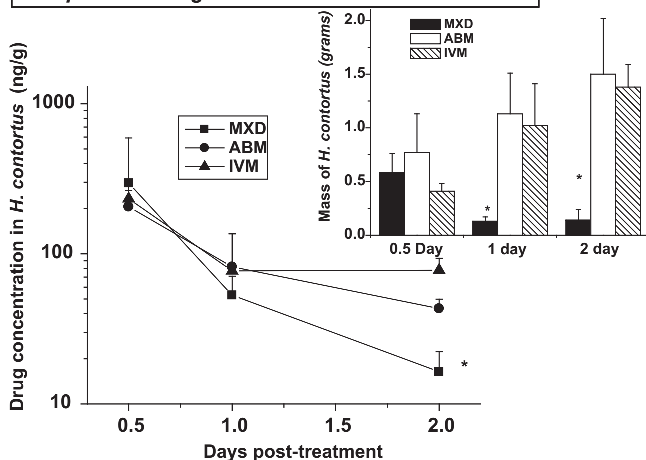

Fig. 3. Comparative mean (±SD) ($n = 4$) moxidectin, (MXD), abamectin (ABM) and ivermectin (IVM) concentrations measured within adult *H. contortus* recovered from intaruminally (IR) treated (0.2 mg/kg) infected lambs. (*) Values for MXD are statistically different from those obtained after the administration of both ABM and IVM at $P < 0.05$. The insert shows the total amount (expressed in grams) of *H. contortus* resistant specimens recovered from lambs treated with the different macrocyclic lactone compounds.

Table 4
Nematode egg counts[1] (range) and faecal egg counts reduction percentages (FECRT) in the untreated (control) and in moxidectin (MXD), abamectin (ABM) and ivermectin (IVM) intraruminally treated animals (0.2 mg/kg) infected lambs.

Treatment group	Mean epg[1] (range)		FECRT (%)	UCL	LCL
	Day 0	Day 14			
Untreated group	**3325** (2010–4440)	**4330**[a] (3180–6660)	–	–	–
MXD	**3432** (2610–3945)	**570**[b] (240–960)	**86.8**	**92**	**78**
ABM	**3307** (2490–4155)	**4460**[a] (1200–6000)	**0**	**34**	**0**
IVM	**3285** (2160–4200)	**4620**[a] (1860–12480)	**0**	**53**	**0**

[1] Arithmetic mean of eggs per gram of faeces; UCL: upper confidence limit 95%; LCL: lower confidence limit 95%. Nematode egg counts at day 14 post-treatment with different superscript are statistically different at $P < 0.05$.

did not show any significant modification. The data on the relative quantification of P-gp2 mRNA in resistant *H. contortus* recovered from untreated and treated lambs is shown in Fig. 4.

Table 5
Adult nematode worm counts (geometric mean) and efficacy obtained at 14 days post-administration of either moxidectin (MXD), abamectin (ABM) or ivermectin (IVM) (intraruminally at 0.2 mg/kg) to nematode infected lambs. Nematode worm counts recorded in the untreated control group is also shown.

Parasites	Untreated group Worm counts	MXD	ABM Worm counts(Efficacy)	IVM
Abomasum				
Haemonchus spp.	1276[a]	133[b]**(89.6%)**	769[a]**(39.7%)**	1021[a]**(20.1%)**
Teladorsagia spp.	1709[a]	0[b]**(100%)**	0[b]**(100%)**	5[b]**(99.7%)**
Trichostrongylus axei	118[a](1900–10400)	0[b]**(100%)**	0[b]**(100%)**	0[b]**(100%)**
Small intestine				
Trichostrongylus colubriformis	963[a]	0[b]**(100%)**	0 [b]**(100%)**	6.28[b]**(99.3%)**
Cooperia spp.	171[a]	0[b]**(100%)**	0 [b]**(100%)**	3.08 [b]**(98.2%)**
Nematodirus spp.	959	0[b]**(100%)**	2.32 [b]**(99.8%)**	4.87 [b]**(99.5%)**
Large intestine				
Trichuris spp.	52.8[a]	0[b]**(100%)**	0 [b]**(100%)**	0[b]**(100%)**

Adult nematode counts at day 14 post-treatment with different superscript are statistically different at P < 0.05.

6. Discussion

The plasma pharmacokinetics of the different MLs has been extensively studied in healthy sheep. However, kinetic information emerging from comparative trials in parasite infected animals is scarce (Lespine et al., 2004; Pérez et al., 2006). The comparative kinetic data generated in the current trial working under standardized experimental conditions showed no significant differences in the systemic exposure (measured as AUC values) after the administration of MXD, ABM and IVM to nematode infected lambs. The persistence of MXD concentrations in plasma was longer compared to ABM and IVM. The higher lipophilicity as well as the lower affinity of MXD by P-gp may account for the well established longer persistence of MXD in the animal body (Lanusse et al., 1997; Lespine et al., 2007; Prichard et al., 2012).

High drug concentrations were measured in the GI sites where parasites are located (abomasum and small intestine) after the IR administration of the three MLs to infected lambs. These concentration profiles were significantly higher than those measured in the bloodstream. The highest drug levels were measured at the abomasal and intestinal contents. The ratio between ML concentrations in the digestive contents and mucosal tissues were between 1.08 and 6.9 (abomasum) and between 0.76 and 1.62 (small intestine) for the three ML compounds under study. These values are significantly higher than those obtained when the MLs are administered by the subcutaneous route to sheep (Lifschitz et al., 1999, 2000). The work described here reports for the first time the comparative *in vivo* ML concentrations measured in adult *H. contortus* recovered from infected treated lambs. As it was recently described for IVM (Lloberas et al., 2012), the drug concentrations measured in the abomasal content for the three molecules under investigation reflected the amount of drug accumulated within the adult *H. contortus*. After the administration of IVM by an enteral route (oral, intraruminal), the access of drug to *H. contortus* was substantially greater than that obtained following the subcutaneous administration (Lloberas et al., 2012). This finding may confirm that an advantageous transcuticular pattern of drug uptake may occur for GI located nematodes when lipophilic compounds formulated as a drug solution are orally administered, achieving large availability of soluble drug at the GI lumen. It is interesting to remark that after the administration of the MLs by an enteral route, there is a good correlation between drug concentrations achieved in the bloodstream and those in GI mucosa ($r = 0.78$, $P < 0.0001$). However, that correlation does not exist between the drug profiles in the systemic circulation and those measured in either digestive contents ($r = -0.01$, $P < 0.953$) or *H. contortus* specimens ($r = -0.02$ $P < 0.892$). The concentration profiles measured within the adult *H. contortus* for the three ML molecules intraruminally administered reflected those achieved in the abomasal content with a significant positive correlation ($r = 0.62$, $P < 0.0002$).

IVM and MXD are closely related ML compounds belonging to the avermectin and milbemycin class of anthelmintics, respectively, and although some pharmacological differences exist, early publications suggested that both compounds have similar mechanisms of action and resistance (Conder et al., 1993; Forrester et al., 2004). However, at the therapeutic dose recommended for ruminants, MXD remains more effective against many IVM-resistant nematode species. Drug concentrations in the GI target tissues/contents during the first 2 days post-treatment are relevant for the effectiveness of the MLs against resident worms in sheep. In the current study, although ML drug levels achieved within the targeted nematode parasites were similar at 0.5 and 1 days post-treatment, MXD concentrations were significantly lower at day 2 post-administration compared to ABM and IVM. Despite the lower drug concentrations accumulated in the parasite tissues, the total mass of *H. contortus* specimens recovered from the abomasum was lower after MXD treatment (at 2 day post-administration) and the efficacy (measure a 14 days post-treatment) was significantly higher for MXD compared to both IVM and ABM (Fig. 3, Table 5). The well described differences in the plasma kinetics disposition between MXD and the avermectin-type compounds are insufficient to explain the observed differential pattern of efficacy. It seems that the pharmacodynamic features of each compound may play a relevant role on the activity against resistant nematodes. A differential pattern of interaction at the parasite site of action, namely the glutamated-gated chloride channel, was recently reported (Prichard et al., 2012). The differences on the chemical structure between MXD and IVM may be sufficient to account for a differential binding to the glutamate-gated chloride channel (Prichard et al., 2012), according to the recently proposed IVM binding model (Hibbs and Gouaux, 2011).

Mammalian P-gps are transmembrane proteins which are able to pump a broad range of structurally unrelated compounds out of the cell by an ATP-dependent process (Ling and Thompson, 1974). P-gp activity has been observed in healthy tissues in mammals, particularly in organs relevant to drug disposition kinetics (Lin, 2003). The *in vitro/ex vivo* interaction of the MLs with mammalian P-gp has been evaluated (Laffont et al., 2002; Ballent et al., 2006; Lespine et al., 2007) and the *in vivo* co-administration of MLs with different P-gp modulator agents has been investigated in different animal species (Lifschitz et al., 2002, 2004; Alvinerie et al., 2008). *In vitro* studies provided clear evidence that MXD has a lower affinity by mammalian P-gp compared to avermectin-type compounds (Lespine et al., 2007). P-gp has been described not only in mammals but also in different parasites (Sangster et al., 1999; Prichard and Roulet, 2007). An enhanced P-gp-mediated

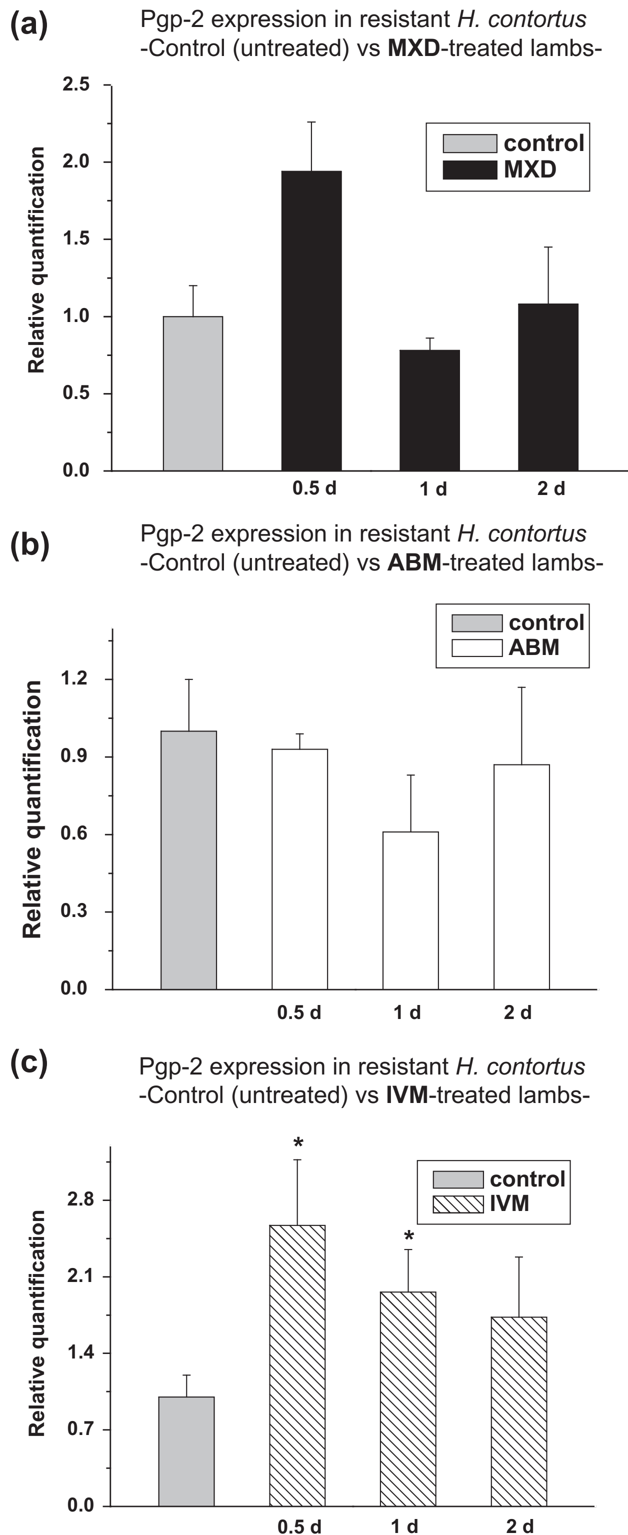

Fig. 4. Relative expression (mean ± SD) of P-glycoprotein 2 (P-gp2) in resistant adult *H. contortus* recovered from untreated lambs and from those intraruminally (0.2 mg/kg) treated with either moxidectin, (MXD) (a), abamectin (ABM) (b) or ivermectin (IVM) (c). (*) Values are statistically different from those obtained in *H. contortus* recovered from untreated lambs at $P < 0.05$.

drug efflux in target parasites has been proposed as a potential IVM resistance mechanism (Xu et al., 1998). In fact, it has been recently shown that modulation of P-gp activity enhances the systemic availability of MLs and improves the *in vivo* field efficacy against resistant nematodes in sheep and cattle (Lifschitz et al., 2010a,b; Bartley et al., 2012). However further investigations are needed to evaluate and understand the potential interaction between MLs and the nematode P-gps. The present study included the assessment of the comparative effects of MXD, ABM and IVM on P-gp2 expression in resistant *H. contortus* recovered from treated lambs at different times post-treatment. Resistant *H. contortus* recovered from untreated lambs showed higher levels of P-gp2 expression than those reported in susceptible strains. Williamson and Wolstenholme (2012) did not find differences in P-gp expression between resistant and susceptibles isolates of *H. contortus*. As that comparison was performed using *H. contortus* larvae (L_3 stage), the changes on the nematode P-gp expression occurring throughout the life cycle could be a confounding factor and may explain this discrepancy (Williamson and Wolstenholme, 2012). The IVM treatment significantly increased the P-gp2 expression in resistant *H. contortus* recovered from treated lambs 0.5 and 1 days post-treatment compared to those parasites recovered from untreated animals. However, treatments with either MXD or ABM did not induce any significant modification in the pattern of the drug transporter expression in the nematode. The up-regulation of P-gp in H. contortus recovered 1 day post-administration was reported previously after IVM treatment, but also to a lesser degree than after MXD administration (Prichard and Roulet, 2007). The reasons to explain the observed differences between IVM and ABM regarding the up-regulation of P-gp2 in *H. contortus* remain unclear, although speculation on the presence of a double bond at C22–23 in the ABM structure (lacking in IVM) may be raised. IVM-induced P-gp up-regulation during a short period of time was also demonstrated in hepatocyte cell lines (Ménez et al., 2012). This induction of P-gp by IVM involved an increase in mRNA half-life in the hepatocytes. The highest level of P-gp induction in the hepatocytes was reached after 24–48 h of IVM exposure and then decreased, which may be in agreement with the observations at the parasite level for the same compound reported here. However, the link between nematode P-gp expression and the MLs accumulation in susceptible and resistant nematodes is not clear and *in vitro* assays should be developed to evaluate the possible role of nematode P-gps in mediating ML resistance. The potential role of P-gps in IVM resistance in *C. elegans* was recently reported (Ardelli and Prichard, 2012). The expression of different nematode P-gps was increased in resistant and susceptible strains in the presence of IVM. In addition, the co-incubation of IVM and P-gp modulators produced significant changes in movement and pharyngeal pumping of the resistant strain of *C. elegans* (Ardelli and Prichard, 2012).

In conclusion, the comparative ML concentrations attained in the target tissues and accumulated within resistant *H. contortus* in lambs was described for the first time in the work described here. The assessment of the drug levels achieved within a target GI nematode in relationship to the obtained efficacy and the expression of a marker of one of the mechanism of resistance, were conducted under standardized experimental conditions in the same infected animals. The knowledge of the epidemiological features of nematode infestations in ruminants together with the pharmacological basis of ML action may supply to practitioners a tool to select compounds suitable for use on farms where the level of susceptibility of nematodes is still adequate.

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