New Zealand Veterinary Journal
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tnzv20

Comparative pharmacokinetic and pharmacodynamic response of single and double intraruminal doses of ivermectin and moxidectin in nematode-infected lambs

M Lloberas[a], L Alvarez[b], C Entrocasso[a], M Ballent[b], G Virkel[b], S Luque[b], C Lanusse[b] & A Lifschitz[b]

[a] Laboratorio de Parasitología, EEA INTA Balcarce, Balcarce, Argentina

[b] Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, BA (7000), Argentina

Accepted author version posted online: 17 Feb 2015.Published online: 18 May 2015.

To cite this article: M Lloberas, L Alvarez, C Entrocasso, M Ballent, G Virkel, S Luque, C Lanusse & A Lifschitz (2015) Comparative pharmacokinetic and pharmacodynamic response of single and double intraruminal doses of ivermectin and moxidectin in nematode-infected lambs, New Zealand Veterinary Journal, 63:4, 227-234, DOI: 10.1080/00480169.2015.1015645

To link to this article: http://dx.doi.org/10.1080/00480169.2015.1015645

Scientific Article

Comparative pharmacokinetic and pharmacodynamic response of single and double intraruminal doses of ivermectin and moxidectin in nematode-infected lambs

M Lloberas*, L Alvarez†, C Entrocasso*, M Ballent†, G Virkel†, S Luque†, C Lanusse† and A Lifschitz†§

Abstract

AIMS: To compare the pharmacokinetics, distribution and efficacy (pharmacodynamic response) of intraruminal ivermectin (IVM) and moxidectin (MXD) administered at 0.2 and 0.4 mg/kg to naturally nematode-infected lambs, and to determine the *ex vivo* accumulation of these anthelmintics by *Haemonchus contortus*.

METHODS: Romney Marsh lambs, naturally infected with IVM-resistant *H. contortus*, were allocated to treatment groups based on faecal nematode egg counts. They received 0.2 or 0.4 mg/kg IVM or MXD (n=10 per group), or no treatment (Control; n=6), on Day 0. Samples from four animals from each treatment group, including abomasal parasites, were obtained on Day 1. Plasma samples were also collected from Day 0 to 14, and a faecal egg count reduction test (FECRT) and a controlled efficacy trial were carried out on Day 14. Concentrations of IVM and MXD in plasma, in abomasal and intestinal tissues and in *H. contortus* were evaluated by high-performance liquid chromatography. Additionally, the *ex vivo* drug accumulation of IVM and MXD by *H. contortus* was determined.

RESULTS: Peak plasma concentrations and the area under the concentration *vs.* time curve for both IVM and MXD were higher for 0.4 than 0.2 mg/kg treatments ($p<0.05$), but there were no differences for other parameters. Concentrations of IVM and MXD in the gastrointestinal target tissues and in *H. contortus* were higher compared to those measured in plasma. Concentrations of both drugs in *H. contortus* were correlated with those observed in the abomasal content ($r=0.86$; $p<0.0001$). The exposure of *H. contortus* to IVM and MXD was related to the administered dose. Mean FECRT and efficacy for removal of adult *H. contortus* was 0% for IVM at 0.2 and 0.4 mg/kg. For MXD, FECRT were >95% for both treatments, and efficacy against *H. contortus* was 85.1% and 98.1% for 0.2 and 0.4 mg/kg, respectively. The *ex vivo* accumulation of IVM and MXD in *H. contortus* was directly related to the drug concentration present in the environment and was influenced by the duration of exposure.

CONCLUSION: Administration of IVM and MXD at 0.4 compared with 0.2 mg/kg accounted for enhanced drug exposure in the target tissues, as well as higher drug concentrations within resistant nematodes. The current work is a further contribution to the evaluation of the relationship between drug efficacy and basic pharmacological issues in the presence of resistant parasite populations.

KEY WORDS: *Ivermectin, moxidectin, pharmacokinetics, efficacy, ex vivo accumulation, drug exposure, resistant Haemonchus contortus*

Introduction

Macrocyclic lactones (ML) are broad-spectrum antiparasitic drugs widely used to control endo- and ectoparasites. Despite the high efficacy initially observed against the most important gastrointestinal nematodes in sheep and goats, resistance to the ML is becoming a serious problem (Kaplan and Vidyashankar 2012). The high level of resistance to ML has encouraged the search for strategies to optimise their great potential as antiparasitic agents in an attempt to extend their life span, particularly in geographical areas where resistance is not yet fully present.

The main strategies proposed to minimise or reverse the impact of drug resistance in nematode control include strategic drug treatment to increase the number of sensitive parasites in refugia, rotation of different chemical anthelmintic groups, combinations of existing different classes of anthelmintics and the limited use of novel compounds (Leathwick and Hosking 2009; Leathwick 2012).

From a pharmacological point of view, anthelmintic drugs need to have the best opportunity to act on the specific site of action of target nematodes (Hennessy 1997). This concept applies to the

AUC	Area under the concentration *vs.* time curve
C_{max}	Peak plasma concentration
FEC	Faecal nematode egg count (s)
FECRT	Faecal nematode egg count reduction test
HPLC	High-performance liquid chromatography
IVM	Ivermectin
ML	Macrocyclic lactone (s)
MXD	Moxidectin

* Laboratorio de Parasitología, EEA INTA Balcarce, Balcarce, Argentina
† Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, BA (7000), Argentina
§ Author for correspondence. Email: adrianl@vet.unicen.edu.ar

http://dx.doi.org/10.1080/00480169.2015.1015645

different strategies addressed to increase drug exposure of parasites. For example, the use of the ML by the oral route in sheep and cattle had greater efficacy against resistant nematodes in comparison to injectable subcutaneous treatment (Gopal *et al*. 2001; Lloberas *et al*. 2012; Leathwick and Miller 2013). The evaluation of ML concentration profiles in the tissues of parasite locations, as well as within target nematodes, greatly contributed to the comprehension of the time course of action for these compounds (Lloberas *et al*. 2013). In this context, the work described here assessed the relationship between anthelmintic activity and parasite exposure to ivermectin (IVM) and moxidectin (MXD) in lambs naturally infected with nematodes. The aim of the current work was to compare the pharmacokinetics, distribution and efficacy of single and double doses of IVM and MXD in nematode-infected lambs, and to determine the *ex vivo* accumulation of these anthelmintics by *Haemonchus contortus*.

Materials and methods

In vivo experiments

The experiments were performed on the experimental farm of National Institute of Agricultural Technology located in Balcarce, Buenos Aires, Argentina. The selected farm is a sheep experimental unit with a parasite control programme based on the intensive use of anthelmintics over the years, where failure of IVM to control nematodes (particularly *H. contortus*) was previously corroborated by faecal nematode egg count reduction tests (FECRT) and controlled efficacy trials (Entrocasso *et al*. 2008; Lifschitz *et al*. 2010; Lloberas *et al*. 2012, 2013). The *in vivo* pharmaco–parasitological experiments were carried out over two consecutive years (2011–2012). The study using IVM was conducted in the first year and that for MXD in the second year.

Ivermectin pharmaco–parasitological trial

Twenty-six Romney Marsh lambs naturally infected with gastrointestinal nematodes were included in this trial. The mean lamb weight was 23.4 (SD 3.38) kg. The selection of the animals was based on faecal nematode egg counts (FEC), as described below. On Day −1 (Day 0 was the day of treatment) all lambs were sampled for FEC, ear tagged and individual bodyweights recorded. The mean FEC of experimental animals was 2,940 (min 1,800, max 5,100) epg. Animals were allocated to a paddock and fed on a lucerne and white and red clover pasture for 20 days before starting the clinical efficacy study and during the experiment. 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 and also following the ethical standards described in the MAF User's Guide (Anonymous 1999).

All lambs were ranked according to FEC and then divided into three groups of animals based on FEC, to perform the pharmacokinetics and efficacy trials. The control group (n=6) remained untreated. Animals in the IVM 0.2 (n=10) and IVM 0.4 (n=10) groups received IVM (Ivomec Oral, Merial, Montevideo, Uruguay) by the intraruminal route at 0.2 and 0.4 mg/kg, respectively. The intraruminal administration was injected percutaneously. Mean initial FEC were 2,970 (min 2,100, max 4,380) epg (Control group), 3,024 (min 1,800, max 4,380) epg (IVM 0.2), and 3,228 (min 2,040, max 5,100) epg (IVM 0.4). The intraruminal route was selected instead of the oral administration to avoid the closure of the oesophageal groove and therefore to minimise the variability.

To study the distribution of IVM in target tissues and parasites, four animals from groups IVM 0.2 and IVM 0.4 were sacrificed on Day 1 and samples of blood, abomasal and small intestine (cranial jejunum) contents and mucosal tissue were collected following the procedures described by Lifschitz *et al*. (2000). From the abomasum of each animal, the total mass of *H. contortus* was recovered to measure the drug concentration in the parasites.

To characterise the efficacy and the plasma disposition of IVM, faecal samples were collected from all the lambs in each experimental group on Days −1 and 14 in order to determine FEC. Jugular blood samples (7 mL) were collected into heparinised vacutainer tubes prior to and at 0, 3, 6, 9 hours and 1, 2, 3, 5, 7, 9, 12 and 14 days post-treatment.

Moxidectin pharmaco-parasitological trial

Twenty-six Romney Marsh lambs naturally infected with gastrointestinal nematodes were used in this trial. The selection of the animals was based on FEC. The mean lamb weight was 21.5 (SD 4.8) kg. Mean FEC of experimental animals was 4,635 (min 600, max 9,721) epg. The experimental conditions and the FEC determinations were similar to those described in the IVM trial. Experimental lambs were assigned into three experimental groups based on FEC. The control group (n=6) remained untreated. Animals in the MXD 0.2 (n=10) and MXD 0.4 (n=10) groups received MXD intraruminally (Cydectin, Fort Dodge, Buenos Aires, Argentina) at 0.2 and 0.4 mg/kg, respectively. The mean initial FEC were 4,202 (min 600, max 5,671) epg (Control group), 4,701 (min 721, max 8,880) epg (MXD 0.2), and 3,881 (min 660, max 9,721) epg (MXD 0.4). Four animals from MXD 0.2 and MXD 0.4 groups were sacrificed on Day 1 post administration and the same samples as in IVM trial were collected. Faecal and blood samples were collected as in the IVM trial.

Blood samples obtained in both trials (IVM and MXD experiments) were centrifuged at 2,000g for 20 minutes and the recovered plasma was kept in labelled vials. Plasma, gastrointestinal mucosal tissues and gastrointestinal content samples were stored at −20°C until analysed by high-performance liquid chromatography (HPLC). Additionally, on Day 14, the remaining animals from all treatment groups were sacrificed by captive bolt gun and rapidly exsanguinated. Abomasum and small and large intestinal sections were identified and isolated and the content analysed to record the different parasite stages following the World Association for the Advancement of Veterinary Parasitology guidelines (Wood *et al*. 1995).

Ex vivo drug accumulation

Three lambs from the same experimental unit, naturally infected with IVM resistant *H. contortus*, were sacrificed and adult specimens of *H. contortus* were collected from abomasum. Adult nematodes were rinsed extensively with saline solution at 37°C to remove the adhering materials, then 20 mg of parasites were placed into khan tubes (Deltalab, Barcelona, Spain) with 1 mL of RPMI medium (R8758, Sigma Chemical, St Louis, MO, USA) and incubated at 37°C. The concentration of ethanol in medium was only 0.1% (v/v) to prevent any harmful effects on the living nematodes. After 30 minutes, IVM and MXD dissolved in ethanol were added to the tubes at a final concentration of 0.5

µM and 5 µM for both drugs. Four replicates were incubated for 15 minutes and 3 hours for each drug. Incubations under similar conditions were performed with dead nematodes. Nematodes were killed by freezing (−20°C, 30 minutes). Blank samples were prepared with medium and nematodes but without IVM and MXD (n=4) and also with medium with drug but without nematodes (n=4). Blank samples contained the same concentration of ethanol (0.1%) and were incubated for the same time intervals. After incubation, nematodes were rinsed thoroughly three times with saline. The nematodes were blotted on coarse filter paper and then transferred to the microtubes. Parasite samples were stored at −20°C until IVM and MXD concentrations were analysed by HPLC.

Analytical procedures

Pharmacological determinations

The extraction of each ML from plasma, tissue and parasite samples was carried out following the technique described by Lifschitz *et al.* (1999, 2000) and Lloberas *et al.* (2012). Basically, 1 mL aliquot of plasma, 0.5 g of gastrointestinal samples (mucosas and contents), 100 mg (*in vivo* trial) and 20 mg (*ex vivo* trial) of parasites were combined with the internal standard compound (doramectin, 10 ng/g for plasma, 40 ng/g for gastrointestinal samples and 200 ng/g for parasites), 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 minutes. Parasites and gastrointestinal tissue/content samples were sonicated in an ultrasonic bath. (Transsonic 570/H, Laboratory Line Instruments Inc., Melrose Park, IL, USA) for 10 minutes. The supernatant was manually transferred into a tube and the procedure was repeated once more for the gastrointestinal 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 derivatisation of ML was carried out with 100 µl of a solution of N-methylimidazole (Sigma Chemical) in acetonitrile (1:1) and 150 µL of trifluoroacetic anhydride (Sigma Chemical) solution in acetonitrile (1:2) (De Montigny *et al.* 1990). After completion of the reaction (<30 seconds), an aliquot (100 µL) of this solution was injected directly into the HPLC system. Concentrations of ML 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/minute at 30°C (Lifschitz *et al.* 1999). A validation of the analytical procedures used for extraction and quantification of each ML from plasma, gastrointestinal mucosa and contents and parasites was performed before starting the analysis of the experimental samples obtained during the pharmacokinetic trial. Calibration curves were established using least squares linear regression analysis, r, and CV calculated. The limits of quantification of IVM and MXD were 0.1 ng/mL or 0.1 ng/g. The percentage of recovery was >70% in the different biological matrices.

Parasitological techniques

The individual FEC were performed using the modified McMaster technique with a sensitivity of 30 epg. The anthelmintic efficacy of the treatments was evaluated by FECRT, calculated according to the formula (Coles *et al.* 1992):

$$\text{FECRT}\,(\%) = 100^{*}\left(\frac{1-\text{T}}{\text{C}}\right),$$

where T is the arithmetic mean FEC in the treated group and C is the arithmetic mean FEC in the Control group on Day 14. The 95% CI were calculated as reported by Coles *et al.* (1992). Direct adult nematode counts of animals from experimental groups were determined on Day 14 according to the World Association for the Advancement of Veterinary Parasitology guidelines (Wood *et al.* 1995). The efficacy of each anthelmintic treatment was determined by the comparison of parasite burdens in treated *vs.* untreated animals. The following equation expresses the percentage of efficacy of a drug treatment against a given parasite species (S) in a single treatment group (T) when compared to an untreated control (C):

$$\text{Efficacy} = \left[\frac{\text{Mean of S in C} - \text{Mean of S in T}}{\text{Mean of S in C}}\right] \times 100$$

The arithmetic mean was used in the estimation of anthelmintic efficacy as was suggested by McKenna (1997).

Pharmacokinetic analysis

Plasma concentrations *vs.* time curves obtained after each treatment in each individual animal were fitted using the PK Solutions 2.0 (Ashland, OH, 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) 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 (λz). The elimination half-life was calculated as ln2/λz. Statistical moment theory was applied to calculate the mean residence time (MRT) as follows:

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}}$$

where 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).

Statistical analysis

Faecal egg and nematode counts (reported as arithmetic mean and SD) were compared by non-parametric ANOVA (Kruskal–Wallis test), with dose rate as the explanatory variable. Mean pharmacokinetic parameters for ML were compared using Student's *t*-test. The assumption that the data obtained after treatments have the same variance was assessed. A non-parametric Mann–Whitney test was used where significant differences among SD were observed. The correlation between concentrations of IVM and MXD in abomasal contents and *H. contortus* was evaluated using Pearson's r. 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.

Results

Plasma pharmacokinetics

The concentration of IVM and MXD in plasma after intraruminal administration to lambs at 0.4 and 0.2 mg/kg are shown in Figures 1 and 2. Concentrations of IVM in plasma were below the assay limit of detection after Day 7. The r^2 obtained after fitting the individual plasma concentrations *vs.* time curves was between 0.93–1 (IVM) and 0.82–1 (MXD). The plasma concentration profiles were related to the dose rate administered for both drugs. The main pharmacokinetic parameters for IVM and MXD are shown in Tables 1 and 2, respectively. For both IVM and MXD, mean C_{max} and AUC were higher for the 0.4 compared to 0.2 mg/kg treatments ($p<0.05$), but there were no differences for other parameters.

Distribution to target tissues and parasites

Mean concentrations of IVM and MXD in plasma, gastrointestinal target tissues and *H. contortus* on Day 1 for 0.4 and 0.2 mg/

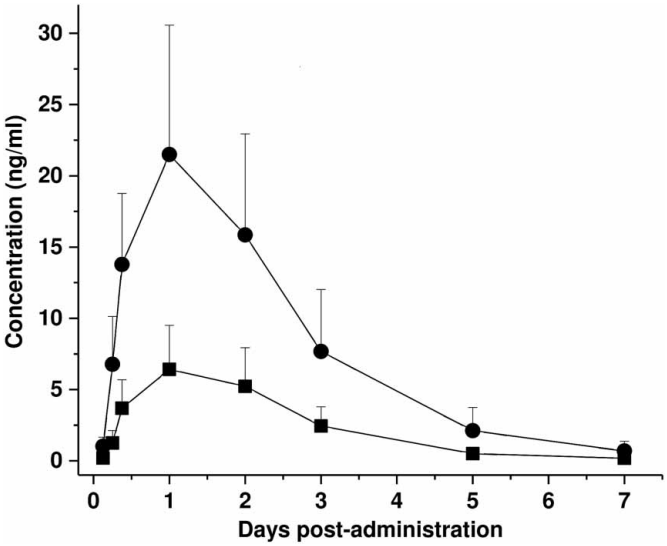

Figure 1. Mean (±SD) concentrations of ivermectin in plasma of nematode-infected lambs after intraruminal administration at 0.2 mg/kg (■n=6) and 0.4 mg/kg (●n=6).

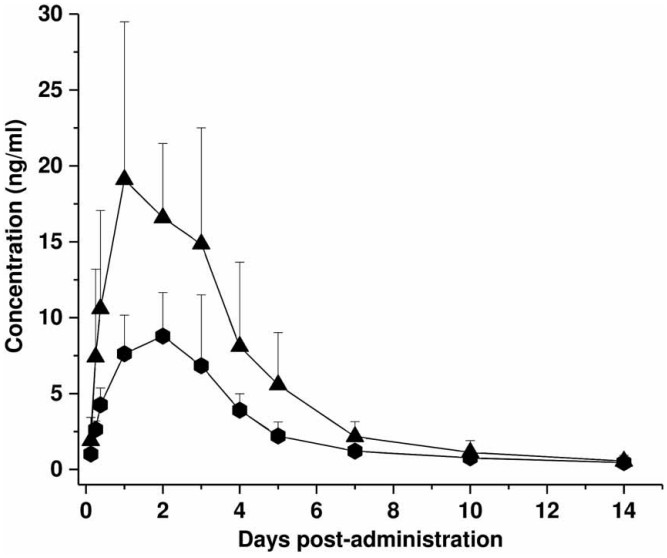

Figure 2. Mean (±SD) concentrations of moxidectin in plasma of nematode-infected lambs after intraruminal administration at 0.2 mg/kg (⬢ n=6) and 0.4 mg/kg (▲n=6).

Table 1. Mean (±SD) pharmacokinetic parameters for ivermectin in plasma of nematode-infected lambs after intraruminal administration at 0.2 mg/kg (n=6) and 0.4 mg/kg (n=6).

Kinetic parameters	0.2 mg/kg	0.4 mg/kg	P-value[a]
$T_{1/2}$ ab (days)	0.36±0.13	0.31±0.13	0.514
T_{max} (days)	1.17±0.41	0.90±0.26	0.198
C_{max} (ng/mL)	6.50±2.98	21.4±9.06	0.002
AUC (ng.d/mL)	17.1±7.60	57.0±27.1	0.004
MRT (days)	2.08±0.23	2.04±0.32	0.793
$T_{1/2}$ el (days)	1.01±0.09	1.23±0.21	0.818

AUC=area under the concentration *vs.* time curve; C_{max}=peak plasma concentration; MRT=mean residence time; T_{max}=time to the peak plasma concentration; $T_{1/2}$ ab=absorption half-life; $T_{1/2}$ el=elimination half-life.
[a] Significance of difference between concentrations of ivermectin.

Table 2. Mean (±SD) pharmacokinetic parameters for moxidectin in plasma of nematode-infected lambs after intraruminal administration at 0.2 mg/kg (n=6) and 0.4 mg/kg (n=6).

Kinetic parameters	0.2 mg/kg	0.4 mg/kg	P-value[a]
$T_{1/2}$ ab (days)	0.48±0.29	0.47±0.32	0.955
T_{max} (days)	2.00±0.89	1.67±1.03	0.563
C_{max} (ng/mL)	10.6±2.54	24.7±3.45	0.001
AUC (ng.d/mL)	39.7±6.06	81.0±16.2	0.002
MRT(days)	4.42±0.72	3.59±1.02	0.135
$T_{1/2}$ el (days)	2.85±0.52	2.17±0.59	0.059

AUC=area under the concentration *vs.* time curve; C_{max} =peak plasma concentration; MRT=mean residence time; T_{max} =time to the peak plasma concentration; $T_{1/2}$ ab=absorption half-life; $T_{1/2}$ el=elimination half-life.
[a] Significance of difference between concentrations of moxidectin.

Table 3. Mean (± SD) concentrations of ivermectin measured in plasma (ng/mL), gastrointestinal mucosal tissues, gastrointestinal contents and *Haemonchus contortus* (all ng/g) on Day 1 after intraruminal administration to nematode-infected lambs at 0.2 mg/kg (n=4) and 0.4 mg/kg (n=4).

	0.2 mg/kg	0.4 mg/kg	P-value[a]
Plasma	8.4±3.62	21.8±9.76	0.042
Abomasal content	395±71.5	585±92.4	0.017
Abomasal mucosa	57.4±8.1	115±16.5	0.001
Haemonchus contortus	102±28	251±137	0.028
Intestinal content	254±53.4	466±159	0.028
Intestinal mucosa	116±52.9	181±11.2	0.028

[a] Significance of difference between concentrations of ivermectin.

Table 4. Mean (± SD) concentrations of moxidectin measured in plasma (ng/mL), gastrointestinal mucosal tissues, gastrointestinal contents and *Haemonchus contortus* (ng/g) on Day 1 after intraruminal administration to nematode-infected lambs at 0.2 mg/kg (n=4) and 0.4 mg/kg (n=4).

	0.2 mg/kg	0.4 mg/kg	P-value[a]
Plasma	13.4±1.94	25.1±2.33	0.001
Abomasal content	355±155	910±395	0.034
Abomasal mucosa	73.9±24.5	166±84	0.028
Haemonchus contortus	209±69	509±179	0.025
Intestinal content	223±116	560±357	0.028
Intestinal mucosa	111±26.8	208±54.4	0.010

[a] Significance of difference between concentrations of moxidectin.

kg treatments are shown in Tables 3 and 4. Concentrations of IVM and MXD in the gastrointestinal target tissues and in *H. contortus* were much higher compared to those measured in plasma, for both treatments. Concentrations of the ML measured in *H. contortus* were correlated with those observed in the abomasal content (r=0.86; p<0.001). Concentrations of IVM and MXD in the gastrointestinal target tissues were higher after administration at 0.4 than 0.2 mg/kg (p<0.05). The exposure of *H. contortus* to IVM and MXD was related to the administered dose; the ratios of drug concentrations in *H. contortus* to abomasal content were between 0.26 and 0.43 (IVM) and 0.56 and 0.59 (MXD). Independent of the ML and the administered dose, the drug concentrations in the gastrointestinal mucosal tissues were lower compared to their respective gastrointestinal contents.

Efficacy trials

The clinical efficacy of IVM and MXD on Day 14 for 0.4 and 0.2 mg/kg treatments is shown in Tables 5 and 6. The mean FECRT was 0% for IVM after administration at 0.2 and 0.4 mg/kg and mean efficacy for removal of adult *H. contortus* was also 0% at both dose rates. In contrast, the mean FECRT for MXD was 96.5% and 98.9% after administration at 0.2 and 0.4 mg/kg, respectively, and mean efficacy against this IVM-resistant strain of *H. contortus* was 85.1% (0.2 mg/kg) and 98.1% (0.4 mg/kg). Mean efficacies against other abomasal and small intestine nematode species were between 89 and 100% after treatment with each of the ML.

Ex vivo accumulation

The *ex vivo* accumulation of IVM and MXD in *H. contortus* was directly related to the drug concentration present in the environment where the parasite was located, and was influenced by the duration of exposure. Mean concentrations of IVM in *H. contortus* after incubation with IVM at 0.5 µM for 3 hours was 53 (SD 25) pmol (46.4 ng) compared with 586 (SD 215) pmol (512 ng) after incubation at 5 µM (p<0.05). After 15 minutes of incubation at 0.5 µM, the total amount of IVM measured in *H. contortus* was 10 (SD 3.9) pmol (8.75 ng), less than that after 3 hours (p<0.05).

Similarly, mean concentrations of MXD in *H. contortus* after incubation with MXD at 0.5 µM for 3 hours was 42 (SD 3.2) pmol (26.9 ng) compared with 325 (SD 42) pmol (209 ng) after incubation at 5 µM (p<0.05). After 15 minutes of incubation at 0.5 µM, the total amount of MXD measured in *H. contortus* was 5.8 (SD 2.5) pmol (3.71 ng), less than that after 3 hours (p<0.05). Comparing living and dead nematodes, there were no significant differences in IVM and MXD accumulation.

Table 5. Mean (min, max) counts of faecal nematode eggs and nematodes obtained on Day 14 after no treatment (Control, n=6), or intraruminal administration of ivermectin at 0.2 mg/kg (n=6) and 0.4 mg/kg (n=6) to nematode-infected lambs, and the calculated efficacy[a] (95% CI).

	Control	0.2 mg/kg		0.4 mg/kg	
	Mean count (min, max)	Mean count (min, max)	Efficacy (%) with 95% CI	Mean count (min, max)	Efficacy (%) with 95% CI
Faecal nematode eggs (epg)	1,610 (1,080, 2,520)	2,790 (880, 3,300)	0 (0–12)	2,160 (1,020, 3,540)	0 (0–27)
Parasites					
Haemonchus spp.	355 (130, 580)	406 (180, 650)	0 (0–34)	418 (150, 740)	0 (0–33)
Teladorsagia spp.	55 (10, 130)	0[x]	100	0[x]	100
Nematodirus spp.	27 (10, 40)	2 (0, 12)[y]	92.5 (37–99)	0[y]	100
Trichuris spp.	19 (10, 40)	2 (0, 12)[z]	89.1 (0–99)	0[z]	100

[a] Faecal nematode egg count reduction test or percentage reduction in count of different parasite genus relative to controls.
[xyz] Adult nematode counts for each parasite genus differ from control group ([x] p=0.002; [y] p=0.001; [z] p=0.028).

Table 6. Mean (min, max) counts of faecal nematode eggs and nematodes obtained on Day 14 after no treatment (Control, n=6), or intraruminal administration of moxidectin at 0.2 mg/kg (n=6) and 0.4 mg/kg (n=6) to nematode-infected lambs, and the calculated efficacy[a] (95% CI).

	Control	0.2 mg/kg		0.4 mg/kg	
	Mean count (min, max)	Mean count (min, max)	Efficacy (%) with 95% CI	Mean count (min, max)	Efficacy (%) with 95% CI
Faecal nematode eggs (epg)	20,855 (6,840, 50,860)	730 (0, 2,220)[w]	96.5 (87–99)	230 (0, 630)[w]	98.9 (96–100)
Parasites					
Haemonchus spp.	4,117 (780, 6,840)	541 (0, 1,333)[x]	86.9 (70–94)	68 (0, 153)[x]	98.4 (96–99.3)
Teladorsagia spp.	1,523 (270, 2,940)	0[y]	100	1 (0, 4)[y]	99.9 (99–100)
Nematodirus spp.	634 (530, 780)	0[y]	100	1 (0, 7)[y]	99.8 (98.5–100)
Trichuris spp.	34,800 (22,300, 57,770)	13 (0, 40)[z]	99.9 (99.9–100)	20 (0, 50)[z]	99.9 (99.8–100)

[a] Faecal nematode egg count reduction test or percentage reduction in count of different parasite genus relative to controls.
[w] Faecal nematode egg counts differ from control group (p=0.003).
[xyz] Adult nematode counts for each parasite genus differ from control group ([x] p=0.002; [y] p=0.001; [z] p=0.005).

Discussion

The current work evaluated the drug accumulation in host gastrointestinal tissues and in *H. contortus* adult parasites collected after IVM and MXD intraruminal administration at 0.2 mg/kg and 0.4 mg/kg. Increases in concentrations of drug at the site of parasite location accounted for an enhancement of drug concentrations in the parasite. Based on pharmacological principles, all the strategies that maximise drug availability (exposure) at the host–parasite interface may increase the nematodicidal effect. Many factors related to animal physiology, route of administration and formulations have been evaluated as strategies to increase and extend drug presence and therefore enhance the anthelmintic effect (Lanusse and Prichard 1993; Hennessy 1997). When the susceptibility of a given nematode species decreases, oral administration of IVM, abamectin and MXD has shown better performance compared to subcutaneous administration of the same molecules, in different ruminant species (Gopal *et al.* 2001; Lloberas *et al.* 2012; Leathwick and Miller 2013). In the current trial the use of intraruminal administration together with the doubling of the dose rate was evaluated as a strategy to increase clinical efficacy.

The main plasma kinetic parameters such as C_{max} and AUC were significantly greater after the administration of IVM and MXD at 0.4 mg/kg compared to the recommended dose of 0.2 mg/kg. As the elimination process for IVM and MXD was not affected by the administration of the double dose (first-order kinetic principles), the kinetic parameters that reflect the persistence of the drug in the body were not modified (Tables 1 and 2). Another study demonstrated that after administration of IVM by an enteral route (oral, intraruminal), access of drug to *H. contortus* depended on the drug concentrations in the abomasal contents (Lloberas *et al.* 2012). A high drug exposure is obtained in the gastrointestinal contents after oral administration of ML pharmaceutically prepared as solutions. In the current work, concentrations of IVM and MXD in the abomasal content on Day 1 were between 21 and 36-fold higher than those measured in plasma (Tables 3 and 4). There was a positive correlation between concentrations recovered in the abomasal content and those measured in adult *H. contortus,* and higher concentrations of IVM and MXD were measured in the parasites after their administration at 0.4 mg/kg compared to the administration at 0.2 mg/kg. The same dose-relationship was observed in the intestinal content and in the gastrointestinal mucosal tissues. Drug concentrations in the gastrointestinal target tissues/contents during the first 2–3 days post-treatment are relevant for the effectiveness of the ML against resident parasites in sheep (Lloberas *et al.* 2013), so the higher drug accumulation observed within the nematode after the administration of IVM and MXD at 0.4 mg/kg may be useful to increase the efficacy against resistant parasites. Similar results were recently described for the new anthelmintic monepantel after its oral administration to sheep. The high availability in the abomasal content obtained during the first 48 hours post-treatment could facilitate accumulation within the parasite through a transcuticular diffusion process (Lifschitz *et al.* 2014).

As was corroborated in many previous trials, MXD is effective against many IVM-resistant nematode species at the therapeutic dose recommended for ruminants (Barnes *et al.* 2001; Vickers *et al.* 2001; Lloberas *et al.* 2013). In the current trial, similar efficacies were observed for IVM and MXD at 0.2 mg/kg and 0.4 mg/kg against susceptible nematode species (Tables 5 and 6). IVM showed 0% efficacy against the *H. contortus* strain at the recommended dose of 0.2 mg/kg. At this dose MXD efficacy was higher (86.7%) than that observed for IVM, but it seems that MXD *in vivo* activity may be decreasing. Interestingly, whereas the double dose of IVM remained ineffective against *H. contortus*, a high efficacy was obtained after the administration of MXD at 0.4 mg/kg (98.2%). Particular pharmacodynamic features for each ML may play a relevant role on the activity against resistant nematodes. A differential pattern of interaction at the glutamated gated chloride channel may support the higher efficacy of MXD (Hibbs and Gouaux 2011; Prichard *et al.* 2012). The relative potency of IVM and MXD against susceptible and resistant nematodes was recently studied using the larval development assay and larval migration inhibition assay (Demeler *et al.* 2013; Kotze *et al.* 2014). In agreement with the *in vivo* results obtained in the current trial, IVM and MXD showed the same potency against susceptible *H. contortus*. However, MXD was more toxic than IVM against resistant isolates of *H. contortus* using the larval development assay (Demeler *et al.* 2013; Kotze *et al.* 2014). On the other hand, the higher potency of MXD compared to IVM against resistant nematodes, was not observed after the incubation of resistant *H. contortus* with IVM and MXD using the larval migration inhibition assay (Demeler *et al.* 2013). Despite the fact that *in vitro* assays with nematode larvae have many logistic advantages, there are discrepancies with the *in vivo* trials that should be elucidated. Overall, it seems that pharmacodynamic features, physic–chemical properties/pharmacokinetic behaviour and differences in the mechanism of resistance may together explain the *in vivo* increased MXD potency (Prichard *et al.* 2012; Demeler *et al.* 2013; Lloberas *et al.* 2013).

The way a drug enters into nematodes is relevant to the efficacy of anthelmintic compounds. To corroborate the *in vivo* results, the accumulation of both compounds was also evaluated *ex vivo*. The adult nematode incubation at 0.5 µM of IVM and MXD reflected the *in vivo* concentrations of both drugs measured in the abomasal contents after administration at 0.2 mg/kg to sheep. There were concentration and time effects on the accumulation of IVM and MXD in *H. contortus.* A significantly higher amount of both drugs was measured within the parasite after incubation with 5 µM compared with 0.5 µM. Also, incubation over 3 hours resulted in a higher accumulation of both drugs (5–7 fold) compared to accumulation over 15 minutes. The *ex vivo* uptake of benzimidazole drugs by nematodes, trematodes and cestodes has been extensively studied in our laboratory (Alvarez *et al.* 1999, 2001; Mottier *et al.* 2006). Drug lipophilicity and the permeability of the helminth's external surface determine the effective concentrations that reach the site of action. Recently the uptake of flubenzadole by *H. contortus* was evaluated (Bartikova *et al.* 2012). The passive diffusion was corroborated as the main mechanism in flubendazole influx and efflux from this nematode. There are scarce data available in the literature on *ex vivo* accumulation of ML in adult *H. contortus*. The effects of ML on adult parasite motility using susceptible and resistant adults of *H. contortus* were recently evaluated. The somatic neuromuscular system was identified as the major site of action and resistance to these drugs (Kotze *et al.* 2012; Demeler *et al.* 2014). The findings from the current trial confirm that the increased drug exposure for both IVM and MXD accounted for an enhanced amount of drug recovered within the target parasite, which was observed in both the *ex vivo* and *in vivo* experiments. However, as the *ex vivo* accumulation assays measured the drug concentration in the

whole parasite, this method did not supply information about potential mechanisms of resistance that may reduce the access of the drug to the site of action. This methodological limitation may explain the similar drug concentrations measured in the dead and live *H. contortus* measured in the *ex vivo* assay. Besides, this method demonstrates the difficulties in reflecting the *in vivo* conditions of the gastrointestinal tract, where the drug is distributed between the mucosal tissue, the particulate material of digesta and the gastrointestinal fluid.

The development of anthelmintic resistance to ML in several nematode species limits the intensive use of these anthelmintics (Leathwick and Miller 2013). Different pharmacological strategies were evaluated to improve the performance of antiparasitic drugs. The success of those strategies may depend on the animal species and the predominant genera of nematodes. The efficacy of oral administration of IVM and MXD was higher than subcutaneous injection against *H. contortus* in sheep and *Cooperia oncophora* in cattle (Leathwick and Miller 2013; Lloberas *et al.* 2013). Nevertheless, subcutaneous treatment gave the highest efficacy for both MXD and abamectin against the *Ostertagia*-like parasites in deer (Mackintosh *et al.* 2014). The degree of susceptibility of nematode populations is a relevant topic to determine the impact of increasing local drug exposure. If the response to a drug is in excess of 80% of its maximum, the final effect will be insensitive to further changes in drug concentrations (Holford and Sheiner 1981). If a clinical study is conducted with animals infected with a susceptible population of parasites, then the therapeutic success achieved after exposure to increasing concentrations of the drug could be indistinguishable. However, if animals are infected with nematodes displaying reduced susceptibility, the clinical response to increased drug exposure at the site of action may be increased (Martinez 2014).

In conclusion, the administration of IVM and MXD at 0.4 compared with 0.2 mg/kg accounted for enhanced drug exposure in the target tissues as well as higher drug concentrations within resistant nematodes. Given the extremely high degree of resistance of the *H. contortus* strain being tested in the current experiment, the administration of a double dose treatment was only effective for MXD. The work reported here is a further contribution to the integrated evaluation of the relationship between basic pharmacological issues and *in vivo* drug activity in the presence of resistant parasite populations.

Acknowledgments

This work was supported by CONICET, Agencia Nacional de Promoción Científica y Técnica (ANPCyT) (PICT 1432) and INTA, all from Argentina.

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Submitted 3 September 2014

Accepted for publication 28 January 2015

First published online 17 February 2015

*Non-peer-reviewed