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*Original article*

# A comparison of the efficacy and pharmacokinetics of ivermectin after spring and autumn treatments against Cyathostominae in horses

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## Abstract

The aim of the present study was to determine the efficacy of ivermectin against Cyathostominae infections and to describe the drug's pharmacokinetic parameters during two seasonal deworming treatments in horses. The study was performed on warm-blooded mares aged 3-12 years weighing 450-550 kg. A single bolus of an oral paste formulation of ivermectin was administered at a dose of 0.2 mg/kg BW in spring and autumn. Fecal samples were tested before treatment and 1, 2, 3, 4, 6, 10, 20, 30, 40, 50, 60, 75 days after treatment. Ivermectin concentrations in blood samples collected before treatment, 0.5, 1, 2, 3, 4, 6, 12, 24, 36 and 48 hours after treatment, and 3, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60 and 75 days after drug administration were determined by high pressure liquid chromatography. Drug absorption was significantly ($p<0.05$) slower ($t_{max}$: 21.89±11.43 h) in autumn than in spring ($t_{max}$: 9.78±8.97 h). Maximum concentrations ($C_{max}$) of ivermectin in the blood plasma of individual horses (8.40-43.08 ng/ml) were observed 2-24 h after drug administration during the spring treatment and 2-36 h (6.43-24.86 ng/ml) after administration during the autumn treatment. Significantly higher ($p<0.05$) ivermectin concentrations were found during the first 4 hours after administration in spring in comparison with those determined after the autumn treatment.

The administration of the recommended dose of ivermectin resulted in 100% elimination of parasitic eggs from feces in spring and autumn treatment.

**Key words**: ivermectin, Cyathostominae, pharmacokinetics, horses

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## Introduction

Nematodes of the subfamily Cyathostominae are the most common equine parasites whose prevalence is estimated at 80-100% of the horse population (Lyons et al. 1999, Gawor 2002, Matthews et al. 2004, Kornaś et al. 2007). The Cyathostominae family includes 50 species referred to as small strongylids (Lichtenfels et al. 2008). Cyathostominae have a simple development cycle during which eggs excreted by infected horses hatch into rhabditiform larvae which become invasive several days later (filariform larvae with preserved sheath). Larvae ingested with grass penetrate the large intestinal mucosa. Molting process takes place in parasitic nodules, and fifth larval stage (L5) nematodes enter the intestinal lumen where they mature. Some of hypobiotic L3 and L4 larvae may remain in the submucosal nodules for several months. Adult parasites reach 4.8 to 13 mm in length, depending on the species. The prepatent period for the original invasion is from 8 to 20 weeks, and the excretion of eggs is continuous.

Whereas the threats posed by *Strongylus* spp. are generally recognized, the pathogenicity of small strongylids remains underestimated. The elimination of other internal equine parasites through periodic antiparasitic treatments has increased the clinical significance of Cyathostominae (Lyons et al. 1999). Acute clinical symptoms caused by mature nematodes are rarely seen, but larval cyathostominosis may involve colic and persistent diarrhea and emaciation with possibly lethal consequences (Love et al. 1999). The progressive resistance of small strongylids to antiparasitic drugs poses a growing problem for horse owners (Chapman et al. 1996, von Samson-Himmelstjerna et al. 2007, Traversa 2010).

Avermectins are a series 16-membered macrocyclic lactone derivatives with potent anthelmintic and insecticidal properties (Pitterna et al. 2009). In recent years in Poland, as in other countries, avermectins are the most widely used drugs as they provide effective protection against the main groups of nematode parasites affecting horses (Lanusse et al. 2009), high degree of safety and slow development of parasitic resistance. Effective programs for controlling parasitic infections in horses require extensive knowledge of the pharmacokinetic properties of avermectins. Plasma disposition kinetic parameters of ivermectin administered orally to horses have been examined by many authors (Perez et al. 1999, 2002, 2003, Gokboult et al. 2001, 2010), but the drug's antiparasitic effects have not been investigated in most of these studies.

Reports on the emergence of Cyathostominae resistant to ivermectin have prompted us to investigate the pharmacokinetics of the drug and its efficacy in spring and autumn deworming treatments in North-Eastern Poland.

## Materials and Methods

### Experimental animals

The study was conducted on nine warm-blooded mares aged 3-12 years, with body weight of 450-550 kg, kept in a herd of 24 horses. In spring and summer, the horses were kept in a pasture for 24 h/day with free access to forage and water. In autumn and winter, the animals were housed in individual boxes with access to a shared paddock for 5 hours a day. The horses were fed hay *ad libitum* and received one daily ration of oats outside the pasture period.

In spring 2010, feces samples were collected from each horse in the herd for parasitological examination. Nine mares excreting the largest number of eggs (250-700/1 g feces) were selected for further study.

### Treatment and sampling

A single bolus of an oral paste formulation of ivermectin for horses (Grovermina, 1% w/v, Biowet Drwalew, Poland) was administered at a dose of 0.2 mg/kg BW over the back of the tongue during spring (May) and autumn (November) treatment.

Fecal samples of 200 g were collected directly from the rectum into plastic containers before treatment and 1, 2, 3, 4, 6, 10, 20, 30, 40, 50, 60 and 75 days after the administration of ivermectin.

Blood samples were collected before treatment, 0.5, 1, 2, 3, 4, 6, 12, 24, 36 and 48 hours after treatment, and 3, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60 and 75 days after the administration of ivermectin. Blood samples (5 ml) were collected by venal puncture from the jugular vein into heparinized tubes (Vacutes Kima, Italy). After centrifugation (2500×g for 10 min. at 4°C) plasma was separated and stored at -80°C until ivermectin analysis by high pressure liquid chromatography (HPLC).

The experimental protocol was approved by the Local Ethics Committee for Animal Experimentation in Olsztyn, Poland (Permission No. 38/2009).

### Parasite analysis

Three samples of 1 g each were acquired from every feces sample and analyzed by the McMaster egg counting technique with the use of Darling's solution (50% saturated saline solution and 50% glycerol)

(Whitlock 1948). The number of parasite eggs was counted for each horse, and the results were presented as the means of three measurements. Eggs were identified according to the method proposed by Ziomko and Cencek (1999) based on morphological analyses (shape, thickness and structure of the eggshell and number of blastomeres) performed under a light microscope at 400 × magnification. Egg size was measured under the Olympus CX31 microscope with a digital camera. The Quick PHOTO MICRO 2.3 application was used for image acquisition and visualization. Fecal samples were cultured for 7 days at the temp. 37°C and the number of intestinal cells in the obtained larvae was counted to confirm Cyathostominae infection.

## Analytical HPLC procedure

Ivermectin was assayed by HPLC with fluorescence detection after liquid-liquid phase extraction, according to the procedure previously described by Eraslan et al. (2010) with own modifications.

Drug-free plasma samples (500 µl) were spiked with the ivermectin standard (Sigma-Aldrich, USA) to reach the final concentrations of 1, 2.5, 5, 10, 20, 50, 75 and 100 ng/ml. Plasma samples (spiked and experimental) were combined with 25 µl of the internal standard (doramectin; 50 ng/ml; Fluka Analytical, USA) and mixed with 750 µl of acetonitrile/ammonia (96:4) (J.T. Baker, USA; Riedel-de Haé, Germany, respectively). After mixing for 10 s, the solvent sample mixture was centrifuged at 2800×g for 10 min (MPW-350R, MPW Med. Instrument, Poland). The supernatants were transferred to 7 ml plastic tubes and 5 ml of chloroform (J.T. Baker) was added. The samples were mixed for 30 s and centrifuged at 2000×g for 10 min. 4 ml of the organic phase was transferred to 7 ml glass tubes and evaporated to dryness at 45°C in a sample concentrator (TurboVap LV Evaporator; Caliper Life Sciences, USA). Dry residues were dissolved in 100 µl of 1-methylimidazole (Sigma-Aldrich) solution in acetonitrile (1:1). To initiate derivatization, 150 ml of trifluoroacetic anhydride (Sigma-Aldrich) solution in acetonitrile (1:2) was added. Finally, 2 µl of the resulting solutions were placed in the HPLC device (Agilent 1100 Series, Agilent Technologies, USA). HPLC analyses were performed using a fluorescence detector (FLD G1321A, Agilent Technologies) at excitation wavelength of 365 nm and emission wavelength of 475 nm with 60:35:5 (v/v/v) acetonitrile, methanol (J.T. Baker) and acetic acid (J.T. Baker), mobile phase (flow rate of 1 ml/min) and C18 column (XBridge, 150 mm × 3 mm, 3.5 µm, Waters, USA).

## Validation

A calibration curve containing eight non-zero standards for each analytical run was prepared. Linearity was assessed for eight calibration points for ivermectin and doramectin. The analysis was conducted in the course of three successive days. Zero and blank plasma samples were also prepared for each analytical run to confirm the absence of interferences. Data was analyzed by the least squares regression method to determinate calibration curve parameters: $y = a \times x + b$. Drug concentrations in plasma samples were determined based on the analyte/internal standard response-peak area ratio × internal standard concentrations. The calculated concentrations were determined from linear regression according to the 1/Y weighting method. Accuracy was established across the range of linearity based on the results of a linearity analysis performed in six runs during 3 days. Data from nine runs was used to assess the extraction efficiency of the analytical method. Samples from all standard curve points, extracted according to the developed method, were compared with standard curve samples for each concentration. Stability experiments were performed to evaluate the stability of the working standard solution, freeze, thaw and autosampler. Stability experiments were evaluated with the use of plasma samples containing lowest quality control (LQC; 1 ng/ml), medium quality control (MQC; 50 ng/ml) and high quality control (HQC; 100 ng/ml) concentrations of ivermectin and doramectin.

## Statistical analysis

The fecal egg count was analyzed based on mean values (±SD; n=9) between selected sampling days in spring and autumn and between the same days in different seasons. Ivermectin concentration-time (C-T) profiles in the studied groups were analyzed based on mean values (±SEM), and the concentrations at each sampling time point were compared between spring and autumn. The analysis was performed using Student's t-test (GraphPad Prism 3.1), and differences were regarded as significant at $p<0.05$.

The pharmacokinetic analysis was performed using Biokinetica 4.0 software. Plasma concentration-time profiles were calculated with the use of a one-compartment model and selected statistical moment parameters.

Table 1. Pharmacokinetic parameters (mean ± SD; n=9) of ivermectin administered orally at a dose of 0.2 mg/kg BW in spring and autumn treatments.

| Parameter | Spring treatment | Autumn treatment | P value |
|---|---|---|---|
| $C_{max}$ (ng/ml) | 21.95 ± 12.86 | 13.30 ± 5.00 | 0.078 |
| $t_{max}$ (h) | 9.78 ± 8.97 | 21.89 ± 11.43 | 0.024* |
| $AUC_{(o-t)}$ (mg/l×h) | 1108.97 ± 326.56 | 1154.87 ± 304.29 | 0.761 |
| $AUMC_{(o-t)}$ (mg/l×h^2) | 73809.36 ± 2539.19 | 98625.28 ± 47751.25 | 0.139 |
| $t_{0.5beta}$ (h) | 105.91 ± 40.64 | 117.84 ± 93.47 | 0.730 |
| $MRT_{(o-t)}$ (h) | 68.66 ± 11.04 | 77.09 ± 28.74 | 0.423 |
| $Vd_{(area, o-t)}$ (l/kg) | 0.30 ± 0.15 | 0.32 ± 0.22 | 0.825 |
| $Cl_{(b) (o-t)}$ (l/h×kg) | 1.35 ± 1.07 | 1.71 ± 0.97 | 0.465 |

* – values significantly different (p<0.05) at the same time pint of blood collection.

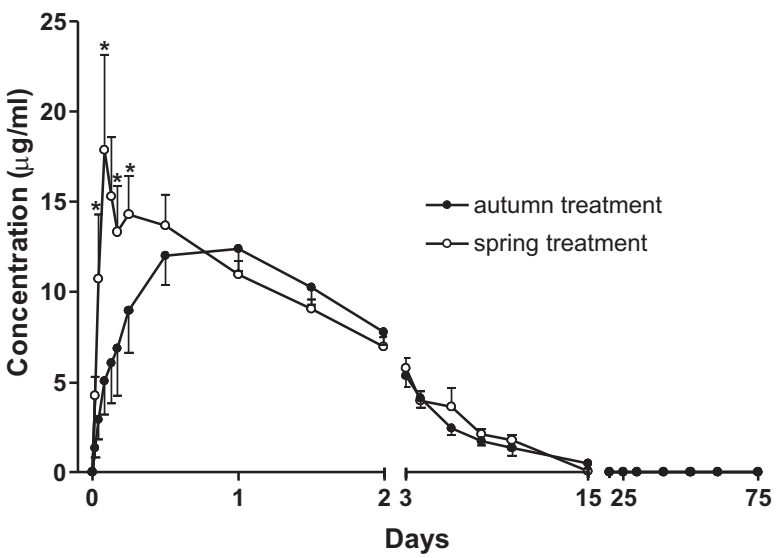


Fig. 1. Mean (±SEM; n=9) plasma concentrations (ng/ml) of ivermectin administered orally at a dose of 0.2 mg/kg BW in spring and autumn treatments.
* – values significantly different (p<0.05) at the same time pint of blood collection.

## Results

The analytical method for extracting, derivatizing and quantifying plasma concentrations of ivermectin and doramectin by chromatographic analysis with fluorescence detection was successfully validated. Calibration curves were plotted using peak area as a function of analyte concentrations. The regression lines between peak areas and drug concentrations produced correlation coefficients in the range of 0.99927 to 0.99989. The limit of quantification, defined as the lowest concentration with a coefficient of variation of < 20%, was determined at 0.99 ng/ml. The mean extraction recoveries from plasma were 75.4%±5.58% and 62.4±4.86% for ivermectin and doramectin, respectively, at spiked concentrations of 1 to 100 ng/ml. The inter-assay coefficient of variability was determined at 6.4%.

The mean pharmacokinetic parameters of ivermectin after spring and autumn deworming treatments are shown in Table 1. Drug absorption was significantly (p<0.05) slower ($t_{max}$: 21.89±11.43 h) in autumn than in spring ($t_{max}$: 9.78±8.97 h). Maximum concentrations ($C_{max}$) of ivermectin in the blood plasma of individual horses (8.40 – 43.08 ng/ml) were observed 2-24 h after drug administration during the spring treatment and 2-36 h (6.43-24.86 ng/ml) after administration during the autumn treatment. The mean plasma C-T profiles of ivermectin after spring and autumn treatments are compared in Fig. 1. No significant differences (p=0.15) in profiles were observed between treatments, but an analysis of sampling time points revealed significantly higher (p<0.05) drug concentrations 4 hours after ivermectin administration in spring than in autumn. No significant differences in the values of the remaining parameters were noted between groups.

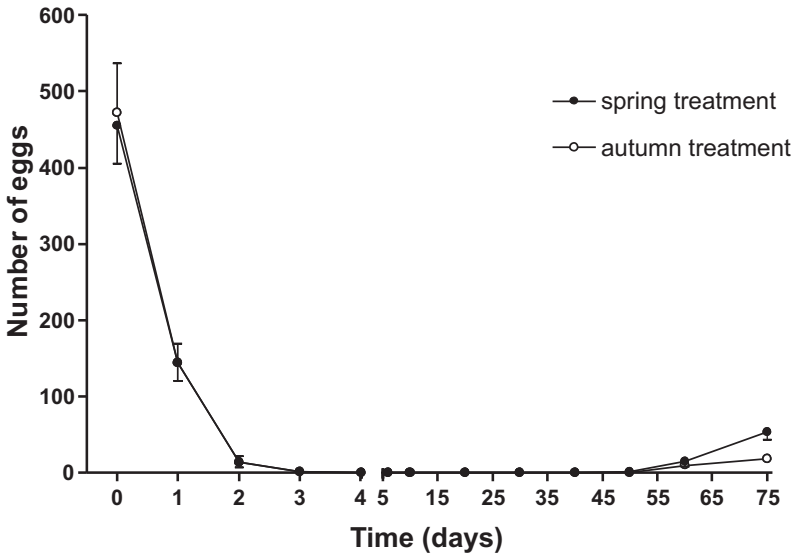


Fig. 2. Average number of parasite eggs in 1 g of horse feces (mean±SD; n=9) before and after spring and autumn treatments involving ivermectin doses of 0.2 mg/kg BW.

Before the spring treatment, average egg counts were determined at 450±150.9 (250-700) eggs per 1 g of feces in 9 horses. All of the observed eggs were oval, thin-walled, 90-122 × 45-70 micrometers in size, and filled with 8 to 16 dark, grainy blastomeres. After 7 days of incubation, the larvae were approximately 850 µm in size, they comprised eight intestinal cells and moult tail of about 3/4 body length, which was indicative of a Cyathostominae infection.

Excreted parasites were present in feces 24 hours after ivermectin administration, and the number of eggs was significantly lower ($p<0.01$) with an average of 150±72.6 eggs (50-250) per 1 g of feces. Four days after the administration of the drug, dead nematodes and eggs were not observed in the feces of any of the examined horses. The presence of individual eggs was determined on treatment day 50 in one mare, on day 60 in 5 horses and on day 75 in the feces of all animals tested (Fig. 2).

The analysis of fecal samples collected from the same 9 horses after autumn treatment revealed an average of 470±195.4 (300-900) Cyathostominae eggs per g of feces. Excreted nematodes were present in feces 24 hours after ivermectin administration, and the number of eggs was significantly lower ($p<0.01$) with an average of 145 eggs (50-250) in 1 g of feces. Four days after the administration of the drug, parasitic eggs were not observed in the feces of any of the horses examined. On treatment day 60, the presence of individual eggs was determined in four horses, and on day 75 isolated Cyathostominae eggs were found in the feces of all the animals.

## Discussion

In the group of avermectin compounds, ivermectin is a drug with the most widely documented pharmacokinetic profile. In our study, the average ivermectin concentrations in the first hours after administration were higher and were achieved significantly faster in spring than in autumn. Our results indicate that dietary habits can significantly affect the rate of absorption immediately after drug administration. An absence of significant differences in other pharmacokinetic parameters may indicate that the time of treatment and/or the severity of infection did not significantly influence those parameters. In this study, the values of $C_{max}$, $t_{max}$ and MRT reported were similar to those observed by Kowalski et al. (2004) at 18.3 ng/ml, 24.24 h and 58.5 h, respectively. In our experiment, the value of $C_{max}$ was half that determined by Pérez et al. (1999) whereas the values of $t_{max}$ and $t_{0.5beta}$ noted by the cited authors were similar to those obtained after the spring treatment in our study. Higher values of $C_{max}$ (51.3 ng/ml) were also reported by Pérez et al. (2002) whereas $t_{max}$ (3.6 h) and $t_{0.5beta}$ (69.36 h) were below the values noted in our study in both treatment periods. Gokbulut et al. (2010) treated infected horses with identical doses of orally administered ivermectin and reported several-fold higher values of $C_{max}$ (61.28 ng/ml) and MRT (176.16 h), somewhat higher values of $t_{0.5beta}$ (156.72 h) and lower values of $t_{max}$ (4.08 h). Pérez et al. (2003) examined different routes of ivermectin administration and observed higher values of $C_{max}$ (51.3 ng/ml) and MRT

(100.84 h) and lower values of $t_{max}$ (3.6 h) and $t_{0.5beta}$ (70.32 h) after oral treatment, but similar values of $t_{0.5beta}$ (108.48 h), several-fold higher values of $t_{max}$ (84 h) and three-fold higher values of MRT (208.4 h) after intramuscular drug administration in comparison with our results. The majority of reviewed studies investigated the pharmacokinetics of ivermectin administered orally to healthy horses (Pérez et al. 2002, 2003, Kowalski et al. 2004), therefore, the differences in pharmacokinetic parameters noted in our study could be partially explained by a Cyathostominae infection. The variations observed can also be attributed to differences in diet and the weight and/or breed of animals.

In a study of yaks administered ivermectin subcutaneously, the values of $C_{max}$ (48.93 ng/ml) were two-fold higher, the values of $t_{0.5beta}$ and $t_{max}$ were similar and the values of MRT were somewhat higher (85.68 h) (Dupuy et al. 2003) in comparison with the pharmacokinetic parameters determined after spring treatment in our study. In a study of dogs that received an identical dose of the analyzed drug orally, the values of $C_{max}$ (116.8 ng/ml) were nearly six-fold and nine-fold higher that those reported in our study after spring and autumn treatments, respectively. The values of parameters such as $t_{max}$ (5.52 h) and $t_{0.5beta}$ (79.68 h) reported in studies of dogs were lower than those noted in horses (Gokbulut et al. 2006). The above findings clearly indicate that the pharmacokinetic profile of ivermectin also varies across different species of animals.

Different deworming treatments are used in veterinary practice to control parasite infections in horses. Some programs offer treatment to all horses which use the same pasture at the same time, regardless of the severity of the parasitic infection, in spring (before the pasture season) and in autumn (after the pasture season) (Reinemeyer and Henton 1987, Lyons et al. 1999) In a different treatment scheme, antiparasitic drugs are administered more frequently, every 8-10 weeks or even daily (Drudge and Lyons 1989, Bello 1996, Reinemeyer and Clymer 2002). According to many researchers (Demeulenaere et al. 1997, Lyons et al. 1999, Gawor and Kita 2006) to prevent the development of drug resistance, antiparasitic drugs should be administered only to horses in whose feces the number of excreted eggs exceeds the „safe" margin (40-200 per 1 gram of feces).

The administration of the recommended dose of ivermectin resulted in 100% elimination of parasitic eggs from feces within four days of treatment. Despite differences in serum drug concentrations between spring and autumn treatments, a comparable clinical effect was achieved. Nematodes were excreted in the periods similar to those observed by other authors (Demeulenaere et al. 1997, Abbot et al. 2003, Osterman Lind et al. 2003, Steinbach et al. 2006). No significant differences were observed in the egg reappearance period (ERP) which was estimated at 9-10 weeks in both seasons, and the above is even longer compared with the findings of other authors (Bello 1996, Demeulenaere et al. 1997, Reinemeyer and Clymer 2002).

Demeulenaere et al. (1997) suggested that the egg reappearance period should be redefined as „the period after antihelmintic treatment during which no eggs or small but acceptable numbers of eggs are excreted". The proposed definition of ERP supports longer intervals between consecutive dewormings, in particular in winter months when horses receive dry feed and are less likely to ingest invasive larvae of small strongylids.

In our study, the season of treatment and differences in animal nutrition had no significant influence on most pharmacokinetic parameters of ivermectin which was administered orally to horses. Our findings also indicate that ivermectin can be effectively used to control equine cyathostominosis in North-East Poland.

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