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Potential Diffusion of Doramectin into a Soil Amended with Female Pig Manure. A Field Experiment

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ABSTRACT: Doramectin is a veterinary drug used as an antihelminthic and is excreted mainly in the feces as the nonmetabolized drug. This study investigated the time profile of doramectin excretion in pig feces and the potential transfer and persistence of doramectin in the soil when the pig manure is used as an organic amendment to the soil. The concentration of doramectin in feces peaked at 143.0 ng/g in the dry feces 4 days after treatment. On day 62, the drug was still detected in the pig feces. After the land application of pig manure, the maximum concentration of doramectin in soil (ppb level) was detected 6 days after treatment. Seven months after the manure application, traces of doramectin were detected in the soil from the surface to a depth of 90 cm. Successive applications of manure from pigs treated with doramectin in a specific area could produce an accumulation of this drug in the soil.

KEYWORDS: doramectin, pig feces, excretion profile, soil, diffuse pollution, HPLC

INTRODUCTION

Veterinary medicines are widely used in treating cattle and can be released into the environment, either directly in feces or urine or indirectly after the application of the manure as an organic fertilizer. Numerous veterinary medicines, such as hormones, antibiotics, and antiparasitics, have been detected in soil, surface water, and groundwater.[1−3] As emerging contaminants, these feces-borne drugs are suspected of causing adverse effects in both humans and wildlife. In the past few years, several studies on the potential impact of veterinary medicines on the environment and on animal health have been conducted.[4−7]

Avermectins (e.g., ivermectin, abamectin, doramectin, milbemycin, eprinomectin, and selamectin) are macrolide endectocides that are now widely used in veterinary medicine because of their broad-spectrum activity against ecto- and endoparasites, their high efficiency, and their high safety margin.[8−10] These drugs are macrocyclic lactones with antihelminthic properties derived from the soil microorganism *Streptomyces avermitilis*. The most frequently used avermectins are ivermectin, abamectin, and doramectin. Doramectin was selected from previous studies because it was the best of several novel avermectins prepared using mutational biosynthesis.[11]

Avermectins are excreted mainly through feces as nonmetabolized drug, and their excretion profile depends strongly on the drug formulation, dosage, animal species, and sex of the animal.[6,12] Pfizer[13] studied the fecal excretion of doramectin for 56 days in treated female and castrated cattle and found that the excretion was approximately 38%, with the maximum excretion levels appearing 21 days after treatment. In horses, Gokbulut et al.[14] recorded the highest concentrations of ivermectin and doramectin in samples taken 24 h after oral administration. Kolar et al.[6] detected a similar time profile for abamectin and doramectin excretion in sheep feces, observing maximum levels in the first days after treatment. Limited data on the fecal excretion profile of doramectin in pig feces are available.

The use of cattle residues contaminated with avermectins as an organic amendment to soil could potentially be a source of diffuse pollution with these veterinary medicines in various environmental media, especially in the soil, surface water, and groundwater. Atmospheric contamination is unlikely because avermectins are nonvolatile and have a very low vapor pressure. Avermectins have a low solubility in water (25 μg/L for doramectin[13]) and a strong affinity for lipids and organic matter. Therefore, avermectins could persist in the soil for long periods of time, thus affecting soil biodiversity.[7,15−18]

The use of pig manure as an organic amendment is a common practice in agriculture. Most studies focus on the dissipation of doramectin from feces, and limited data are available regarding the potential diffusion of the drug into the soil after spreading of manure on the land. This paper has two major objectives: to investigate the time profile of the excretion of doramectin in pig feces and to determine the potential transfer and persistence of doramectin in soil when the pig manure is used as an organic amendment.

MATERIALS AND METHODS

Feces from pigs treated with doramectin were collected, and the manure was applied to an agricultural soil as an organic amendment. An analytical method was developed to determine the concentration of doramectin in the soil and pig manure. The analytical method was based on extraction with acetonitrile, cleanup using solid-phase extraction (SPE), and analysis by high-performance liquid chromatography (HPLC) after derivatization using fluorescence detection.

The doramectin excretion profile in pigs was monitored, and the concentration of doramectin in the soil was evaluated for 7 months

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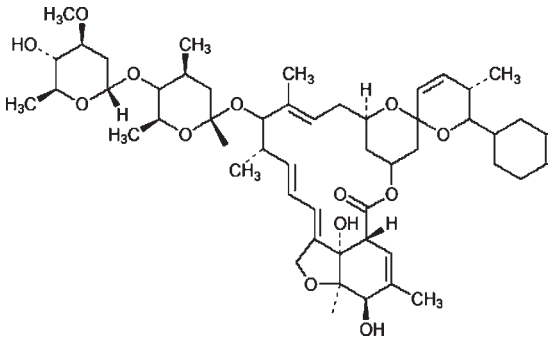

Figure 1. Chemical structure of doramectin.

(from November 2009 to June 2010). The study was conducted at an experimental pig farm located in Aranjuez (Madrid, Spain), where antiparasite treatment of the pigs was usually performed using doramectin.

Chemicals. Acetonitrile and methanol were of HPLC grade and were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Triethylamine (analysis grade, >99%), *N*-methylimidazole, trifluoroacetic acid anhydride (both of GC grade, >99%), and trifluoroacetic acid (HPLC grade, >99%) were purchased from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q system (Millipore, Bedford, MA).

Doramectin (Dr. Ehrenstorfer, Augsburg, Germany) was used as the standard reference material (purity = 90.5%) (see Figure 1 for structure). A stock solution of doramectin at a concentration of 380 μg/mL and working standard solutions (10 and 0.5 μg/mL) were prepared in acetonitrile.

Instrumentation. A Supelco Vacuum Manifold (Bellefonte, PA) and SPE cartridges (C8, 500 mg, 6 mL) from Phenomenex (Torrance, CA) were used for the cleanup procedure. A shaker (Rotaterm, Selecta, Barcelona, Spain) was used to extract the samples, and a Beckman (Fullerton, CA) centrifuge (J2-21) was used to centrifuge the samples. The extracts were evaporated using a Liebisch evaporator (Labortechnik, Bielefeld, Germany).

Extraction and Cleanup. The extraction procedure for doramectin was based on the method described by Kolar et al.[19] with some modifications. Samples of soil (6.0 g) and pig feces (1.0 g) were placed in 50 mL extraction tubes, and 10 mL of acetonitrile was added. The tubes were shaken at room temperature for 30 min and then centrifuged at 13750*g* (15 °C, 15 min). The supernatant was collected, and the residue was extracted again with 10 mL of acetonitrile. The supernatants were mixed and diluted to 80 mL with Milli-Q water containing 0.1% triethylamine (TEA). The mixture was passed through a C8 SPE cartridge previously conditioned with 5 mL of acetonitrile followed by 5 mL of acetonitrile/water (30:70, v/v, 0.1% TEA). After the diluted extract was loaded, the cartridge was washed with 7 mL of acetonitrile/water (30:70, v/v, 0.1% TEA) and then dried under vacuum for 15 min. The analyte was eluted with 6 mL of acetonitrile and evaporated to dryness under a stream of nitrogen at 45 °C. Three replicates were performed for each sample.

Derivatization Reaction. Doramectin is a nonfluorescent molecule; its derivatization to a fluorescent derivative was carried out according to the protocol proposed by Berendsen et al.[20] Briefly, the residue was dissolved in 650 μL of acetonitrile to which 100 μL of *N*-methylimidazole/acetonitrile (1:1), 50 μL of TEA, 150 μL of trifluoroacetic anhydride/acetonitrile (1:2), and 50 μL of trifluoroacetic acid were added. After mixing, the derivative was incubated for 30 min at 70 °C, and finally, the solutions were transferred to glass vials and kept away from sunlight until injection. A standard solution of the doramectin fluorescent derivative was stable for up to 48 h after the derivatization reaction, whereas the stability of the derivatives of the soil and pig manure samples decreased significantly ($p < 0.05$) after 24 h.

Table 1. Physicochemical Properties of the Soil (0−30 cm) from the Olive Grove (Aranjuez, Madrid)

pH	8.1
EC (dS/m)	0.50
organic matter (%)	1.7
total nitrogen (%)	0.1
carbonates (%)	27
phosphorus (mg/kg)	35
Ca (mg/kg)	4161
Mg (mg/kg)	197
Na (mg/kg)	45.5
K (mg/kg)	487
total sand (%)	58
total silt (%)	37
clay (%)	5
texture class, USDA 1994	sandy loam

HPLC Conditions. A Waters HPLC system (Milford, MA) equipped with the following components was used: a gradient pump (600 Controller) equipped with a degasser, a 717 plus autosampler, a column heater, and a fluorescence detector model 2475 Multi λ. An aliquot of 20 μL of derivatized extract was injected on a Phenomenex Luna C18 (2) column (150 × 4.6 mm i.d.; 5 μm particle size) with a Phenomenex C18 precolumn (4.0 × 3.0 mm i.d.; 5 mm particle size). The column temperature was maintained at 35 °C. The mobile phase consisted of methanol, acetonitrile, and water (48:47:5, v/v/v) and was pumped at a rate of 1.2 mL/min. The fluorescence detector was set at an excitation wavelength of 365 nm and an emission wavelength of 470 nm.

Pig Feces Samples. Doramectin excretion in pig feces was evaluated after a single subcutaneous dose (0.3 mg/kg body weight of 1% injectable solution, Dectomax, Pfizer, France). Pig feces samples from untreated pigs were collected to develop the analytical method and were used as a negative control. Forty-one sows (Sach pig, mini-pig) aged between 2 and 6 years and with a body weight between 60 and 70 kg were included in the study. Each pig received approximately 2 kg of feed (Nantaunic, Nanta, Madrid, Spain) per day, which contained 15% crude protein, 8% crude fiber, 3.7% crude fat, 6.2% crude ash, and 0.77% lysine. Water was supplied on demand. The animals lived outdoors and were divided into six pens. The floors were made of cement and covered with straw. Freshly produced fecal matter from the treated pigs was collected in 100 mL sterile plastic vials on day 0 (before administration) and on days 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, 19, 22, 27, 32, 37, 42, 52, and 62. The feces from all of the pigs on each day were combined, homogenized, dried for 28 h at 45 °C, crushed, and sieved (<2 mm). The average moisture content of the feces was 70%, as calculated by weight loss: each pig excreted an average of 1 kg of moist feces, which amounted to 300 g of dry feces.

All of the manure mixed with straw (660 kg) collected until day 11 after veterinary treatment was combined, homogenized, and kept under cover in the field for 8 days. During this time, the doramectin concentration was evaluated on two occasions (at the beginning of the 8 day period and just before application) to determine the evolution of this veterinary drug while the pig feces were stored.

Field Experiments. All of the collected manure was applied to an olive grove on an experimental farm in Aranjuez. Two plots (called A and B (14 × 5 m)) were treated with 4.6 kg/m^2 of manure, according to traditional practices. The field was plowed (to a depth of approximately 30 cm) before treatment. Two equal plots without treatment were used as control soil. In this area, the soil possessed a sandy loam texture, and its characteristics are described in Table 1.

To evaluate the potential transfer and evolution of doramectin into the soil, two soil samples per plot were collected with a soil probe

(each plot was divided into two subplots in which two composite samples were taken), at depths between 0 and 30 cm on day 0 (just before soil fertilization) and on days 1, 6, 29, 63, 85, 115, and 213 after the manure had been applied to the olive grove. To evaluate drug mobility, deeper samples (30−60 and 60−90 cm) were collected on days 115 and 213. Soil samples were air-dried, passed through a 2 mm screen, and mixed prior to analysis to ensure homogeneity.

Soil properties were analyzed according to Ministerio de Agricultura, Pesca y Alimentación (MAPA) guidelines.[21] Electrical conductivity (EC) and pH were measured at a 1:2.5 soil-to-water ratio. Organic matter and total nitrogen content were determined using Walkey−Black[22] and Kjeldahl methods, respectively. Soluble phosphorus was evaluated using the Olsen method.[23] Carbonate levels in the soil were measured using the Bernard calcimeter method.[24] Available nutrients were extracted with 0.1 N NH_4Ac and assessed using atomic absorption spectrometry (AA 240 FS, Varian). Soil texture was analyzed according to the method of Day.[25]

Statistical Analyses. Differences in doramectin contents between the different sampling times were evaluated by one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$ using the SPSS statistical package for Windows, release 16.0. Mean values were compared by Tukey's honestly significant differences (HSD) multiple-range test to detect significant differences. The excretion profile of doramectin in pigs was adjusted to a first-order kinetic equation

$$dC/dt = -kC$$

where C (in ng/g) is the instant concentration of the drug at time t (days) and k (day^{-1}) is the excretion rate constant, which was obtained using linear regression. Microsoft Excel was used to perform this statistical analysis.

RESULTS AND DISCUSSION

Analytical Characteristics of the Chromatographic Method. The calibration curve for doramectin was obtained with external standard calibration at eight concentration levels ranging from 2.5 to 100 ng/mL. The calibration curve showed good linearity, with a coefficient R^2 of 0.999. The limit of detection (LOD) of doramectin in the HPLC system was calculated by injecting solutions containing progressively smaller amounts of doramectin after the derivatization process[26] and was found to be 0.005 ng. In addition, the LOD in samples was calculated as 3 times the signal-to-noise ratio (LOD = 3 S/N) and was found to be 0.2 ng/g for soil and manure samples (dry samples). The limit of quantification (LOQ = 10 S/N) was found to be 0.7 ng/g.

To evaluate the accuracy of the method, the recoveries from blank samples of soil and pig feces spiked with doramectin at four levels (2.5, 7.0, 25, and 42 ng/g (n = 6)) were determined. A doramectin/acetonitrile solution at 0.5 μg/mL was used to spike the blank samples. The samples were in contact with this solution for 30 min before the extraction process was performed. Table 2 shows the mean recoveries, which ranged from 76.8 to 97.0% for soil samples and from 66.7 to 98.8% for manure samples. The reproducibility of the method (Table 2), expressed by the relative standard deviation (RSD), was <10%. The method showed a low detection limit, good reproducibility, and good recoveries from feces and soil and was consistent with the values found in the literature with a similar matrix.[14,19] This method was considered to be applicable to the determination of doramectin in soil and pig feces samples. Typical chromatograms for doramectin standard solution, blank soil and a soil with doramectin are presented in Figure 2.

Doramectin Excretion Profile. Doramectin was not detected in feces collected before veterinary treatment. Figure 3 shows the pattern of doramectin excretion after a single subcutaneous injection at 0.3 mg/kg body weight in pigs. The maximum concentration of doramectin in manure was 143.0 ng/g (dry feces), detected 4 days after treatment. In experiments with sheep, Kolar et al.[6] and Taylor[12] also found that the maximum excretion occurred in the first days after animal treatment. Different results were found in experiments with cattle, for which the maximum excretion was detected 21 days after treatment.[13] A rapid decrease after 9 days was observed, yet the doramectin was still detected on day 62 after treatment (8.8 ± 0.5 ng/g). These results are consistent with the literature data: the doramectin excretion profile depends strongly on the animal species.[6,12−14] The residence time of doramectin in pigs was longer than in sheep. In sheep, the drug concentration was <1 ng/g (dry feces) 42 days after treatment.[6] In horses treated with doramectin, a mean residence time of 3 days was observed.[14]

Table 2. Recoveries (n = 6) and Reproducibility (RSD) of Doramectin in Samples of Soil and Pig Feces

matrix	theor concn (ng/g)	recovery (%) (mean ± s)	RSD (%)
soil	2.5	76.8 ± 5.3	7.0
	7.0	79.9 ± 5.0	6.3
	25.0	96.4 ± 8.3	8.3
	42.0	97.0 ± 2.0	2.1
pig feces	2.5	98.8 ± 1.6	1.6
	7.0	78.1 ± 6.1	7.8
	25.0	66.7 ± 5.3	7.9
	42.0	73.5 ± 4.4	6.0

The elimination profile of doramectin in pigs was adjusted to a first-order kinetics equation, taking the maximum amount of the drug excreted as the starting point. An excretion rate constant, k, of 0.053 day^{-1} was obtained, with a correlation coefficient (R^2) of 0.83. This k value calculated in pig feces was much lower than the value presented in the literature for sheep (k = 0.19 day^{-1});[6] therefore, doramectin excretion in pigs is slower than in sheep.

Because the average dose of doramectin per pig was 0.195 mg, the total amount of doramectin injected into the 41 pigs was 8.0 mg. Over 6 days, the amount of doramectin excreted in the feces was 2.1 mg (26%) (Table 3). Pfizer,[13] in a study on the fecal excretion of doramectin in treated female and castrated cattle, found that the excreted dose was close to 38% after 56 days.

Doramectin in Soil. The pig manure collected up to day 11 after veterinary treatment was stored for 8 days and was then applied to two plots (A and B) in an olive grove. In this application scheme, we evaluated the most critical set of conditions, with the highest concentrations of the drug in the manure. During the 8 day storage period, the doramectin concentration measured in manure did not change significantly ($p < 0.05$), and the average value was 101.4 ± 18.1 ng/g (dry feces); this is equivalent to approximately 33 mg of doramectin per plot.

After the application of the manure to the field, the doramectin levels were monitored over a period of 213 days (Table 4). In control plots, doramectin was not found; the characteristics of the two treated plots, A and B, were similar. Soil samples collected before fertilization treatment (day 0) did not show doramectin. The maximum concentration of doramectin was detected 6 days after treatment (4.04 ± 1.63 ng/g), 2 orders of magnitude below the concentration in the manure. On day 29, the doramectin concentration decreased significantly in soil. Mean values were similar to those found on day 63 in plot A and on day 85 in the two plots. Under these experimental

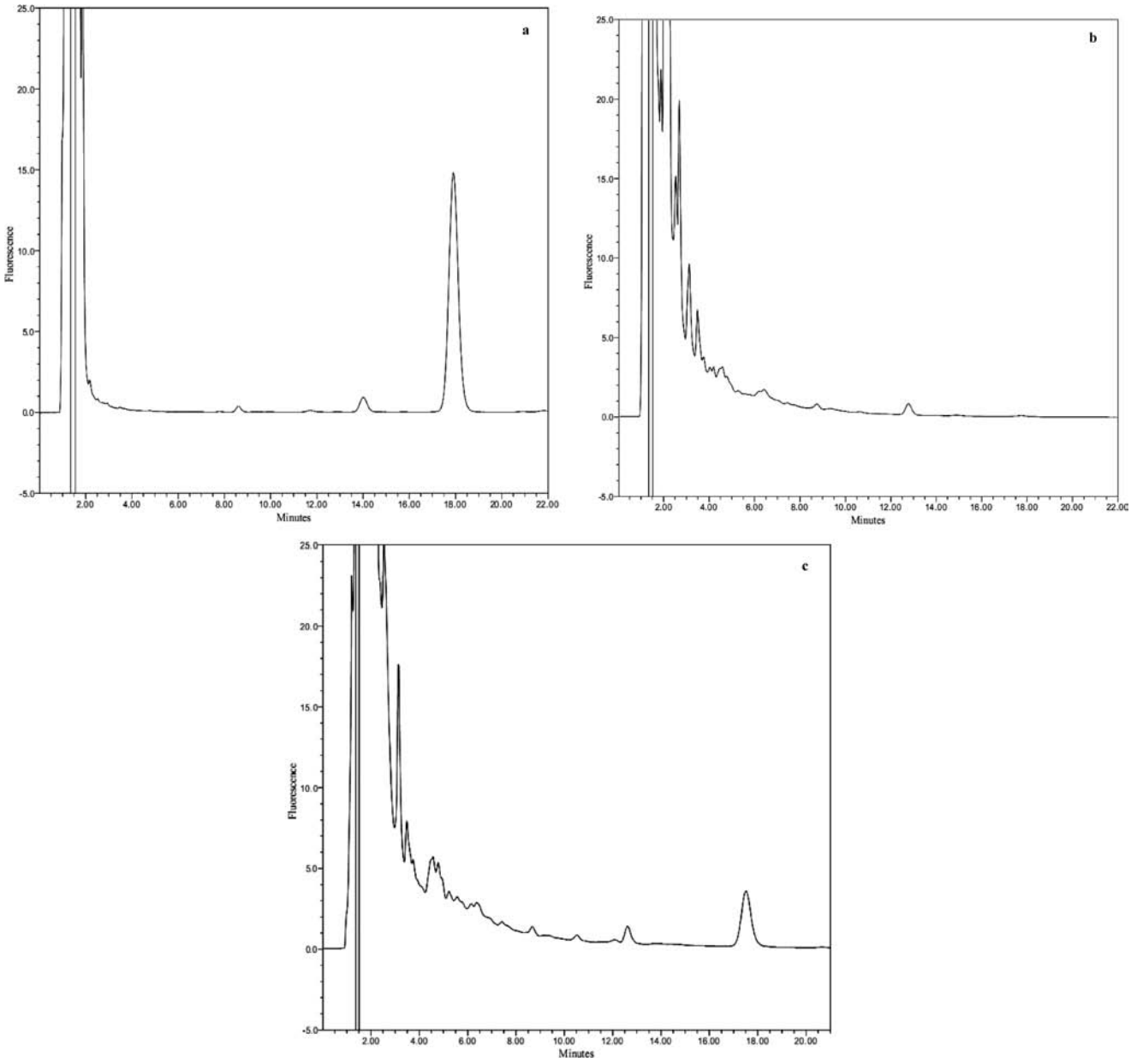

Figure 2. Chromatograms of doramectin standard solution (a), control soil sample (b), and amended soil (c).

conditions, at the highest concentrations of the drug in the manure, doramectin diffusion from manure to soil was moderate, because a low concentration of doramectin in soil was found even after a recent application. There are not many field experiments that evaluate doramectin diffusion into soil to allow us to compare results. Our data are consistent with Taylor's[12] estimates in which 1.8 ng/g of doramectin could be found in soil where sheep treated with doramectin had grazed. Published data on doramectin concentrations related to ecotoxicity effects in soil fauna are higher than the values found in the present study.[16,27] Kolar et al.,[16] in a study of toxicity of doramectin to soil invertebrates, detected negative effects on the body weights of the tested organisms (collembolans, enchytraeids, earthworms) with a concentration of doramectin in soil varying between 8.4 and 100 mg/kg.

On day 115 after soil treatment, the doramectin level was lower than the LOQ at the most superficial zone, whereas the concentration in the soil was close to 1 ng/g at soil levels between 30 and 60 cm for the two plots. In samples from day 213, doramectin was found from the surface to a 90 cm depth, although the amounts were below the LOQ. This result demonstrates some mobility of the doramectin molecules after 7 months because the soil texture was a sandy loam. The mobility of the doramectin is probably associated with the presence of organic matter because doramectin presents a high organic carbon-normalized sorption coefficient (K_{oc}) of approximately 7500 for a silty loam soil.[13]

In general, the mobility of pharmaceutical compounds in soil depends on the drug concentration and source, rain intensity, and soil type.[28,29] Studies about the sorption and mobility of pharmaceutical compounds in soil have shown high mobility in soil organic matter-poor soils, which emphasizes the potential transport of these compounds to groundwater.[28,30] The application of

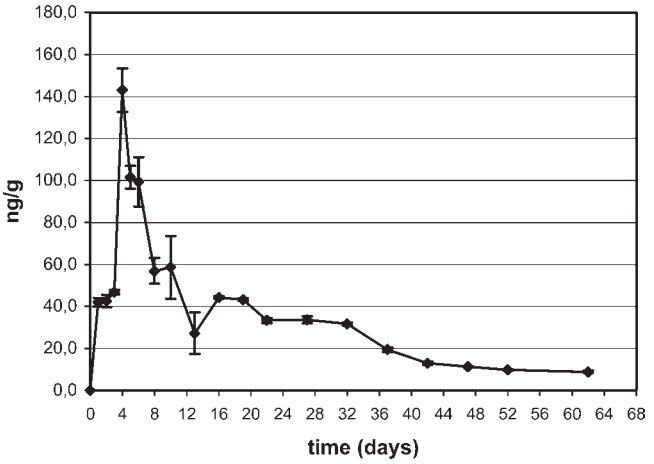

Figure 3. Excretion profile of doramectin (mean value ± standard deviation) in pig feces after a single subcutaneous injection of doramectin (0.3 mg/kg body weight of 1% injectable solution).

Table 3. Estimation of Total Doramectin Excreted per Day

day	doramectin in feces (ng/g)	total doramectin (μg) (μg/g doramectin × 300 g of dry feces × 41 pigs)
0	0.0	0.0
1	42.0	186.4
2	42.6	189.1
3	46.8	207.7
4	143.0	634.8
5	101.5	450.9
6	99.3	440.9
8	56.8	252.4
10	58.6	260.4
13	27.2	120.7
16	44.2	196.1
19	43.2	192.0
22	33.4	148.2
27	33.6	149.4
32	31.6	140.5
37	19.4	86.0
42	13.0	57.9
47	11.2	49.7
52	9.7	43.1
62	8.8	39.1

irrigation water and organic amendments such as biosolids or manure containing pharmaceutical compounds could impose an important risk of introducing drugs into soil and groundwater.

Doramectin is also an un-ionized molecule at environmental pH (5−9). Sorption to soil is therefore not likely to be pH dependent. Boxall et al.,[1] in a monitoring study on the level of veterinary drugs in the environment in the United Kingdom, did not detect doramectin in soil samples, although it was found in sediment samples (2.69 ng/g).

In conclusion, this study shows that pigs excrete the highest levels of doramectin in the feces in the early days after treatment, although doramectin could still be detected in the feces after 60 days. In the field experiment, the application of manure containing doramectin

Table 4. Mean Doramectin Concentration in Soil Samples Treated with Pig Manure

		doramectin concn[a] (ng/g)	
day	depth (cm)	plot A	plot B
0	0−30	nd	nd
1	0−30	0.78 ± 0.07 a	<LOQ
6	0−30	4.04 ± 1.63 b	3.13 ± 2.11 a
29	0−30	0.93 ± 0.21 a	1.06 ± 0.28 b
63	0−30	1.03 ± 0.30 a	<LOQ
85	0−30	1.57 ± 0.40 a	1.15 ± 0.43 ab
115	0−30	<LOQ	<LOQ
	30−60	1.03 ± 0.20 a	0.97 ± 0.21 b
	60−90	nd	nd
213	0−30	<LOQ	<LOQ
	30−60	<LOQ	<LOQ
	60−90	<LOQ	<LOQ

[a] nd, not detected; LOQ, limit of quantification. The same letter indicates mean values ($p < 0.05$) that are not significantly different.

under the specified conditions led to the presence of low levels (<5 ng/g) of the drug in the soil. Seven months after the manure application, traces of doramectin were still detected from the surface of the soil to a 90 cm depth. Successive applications of manure from pigs treated with doramectin in a specific area would produce an accumulation of this drug in the soil that reached toxic levels for soil fauna. Our future research will focus on studying the effects of successive land applications of manure containing doramectin.

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