

## Enrofloxacin and Ciprofloxacin Uptake by Plants from Soil

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**Abstract.** Very small amounts of pharmaceuticals present in everyday food may generate strains of resistant microorganisms in human and animal organisms. This study involves the uptake and accumulation of some widely used fluoroquinolones – enrofloxacin and ciprofloxacin – by plants cultivated in soil augmented with drugs using the microbiological agar diffusion method. *Bacillus subtilis* was used as the test bacterium. The three plants chosen for the experiment were lettuce (*Lactuca sativa*), common barley (*Hordeum vulgare* L.) and cucumber (*Cucumis sativus* L.), which were cultivated in a laboratory in soils mixed with enro- or ciprofloxacin at nominal concentrations of 500, 200, 50 and 10 µg/g. The concentrations of fluoroquinolones remained unchanged in the soil during the experiment. The presence of enrofloxacin was detected in all plants grown at enrofloxacin concentrations of 500, 200 and 50 µg/g. The presence of ciprofloxacin was only detected in barley and cucumber grown in soil with a base concentration of 500 µg/g. In lettuce, which had a longer vegetation period, the presence of ciprofloxacin was detected at all concentrations. The content of ciprofloxacin in the lettuce was 44 µg/g at a soil concentration of 10 µg/g: fluoroquinolones accumulate in a plant during the vegetation period.

**Key words:** enrofloxacin, ciprofloxacin, pharmaceutical residue, plant uptake, microbiological agar-diffusion method, *Bacillus subtilis*

### INTRODUCTION

By the end of the 20<sup>th</sup> century, growing concerns had emerged about the occurrence and fate of pharmaceuticals in the environment, and these concerns have continued to grow steadily ever since (Martínez-Carballo et al., 2009). The reason why pharmaceuticals may become harmful in the environment is that they are designed to affect biological objects. They have lipophilicity, which enables them to permeate bio-membranes, and stability, which prevents their inactivation before the therapeutic effect. Therefore, drugs have the properties they need to accumulate in organisms and cause changes in water and soil ecosystems. The annual consumption of widely used drugs can be quite extensive (Halling-Sørensen et al., 1998). In Denmark, the total annual use of antibiotics is about 34 tonnes (Stuer-Lauridsen et al., 2000).

Generally, drugs can be divided into two groups: drugs used in human medicine

and drugs used in veterinary medicine. Veterinary medicines are used in livestock barns and bird farms as growth stimulators. Livestock are also treated directly on pastures. On fish farms, drugs are introduced into water as a component of feed. Medicines used by humans or animals are excreted in an unchanged form or as metabolites. Drug residue from human excrement reaches the sewage system and finally ends up in sewage treatment plants. An unknown amount of human medicines enter the sewage system as raw sewage.

Depending on the drug, 12–90% of them pass through sewage treatment plants unaltered (Stumpf et al., 1999). It can be concluded that treatment facilities do not remove drug residue completely. A considerable amount of drugs enter surface water and can end up in drinking water. The fate of drug residue after entering a treatment facility may be one of the following: (1) the substance is easily degradable and decomposes fast and fully into CO<sub>2</sub> and water in the facility; (2) the substance is lipophilic and does not degrade easily, instead accumulating unaltered in the activated sludge of the facility; or (3) the substance is metabolised into a more hydrophilic matter but does not degrade at all, instead passing through the treatment plant and entering the aqueous environment.

Sewage sludge containing drug residue is used as a fertiliser in fields (Lillenberg et al., 2009; Radjenović et al., 2009; Lapen et al., 2008). Drugs reach the soil in this way, where they can affect microorganisms and accumulate in plants. Growth stimulators and medicines used in animal breeding reach manure either unaltered or as metabolites, finally entering the fields. On pastures, drugs go through cattle and are excreted. In this manner, extremely high concentrations of drug residue are concentrated locally in the soil, and essentially have a strong impact on soil organisms and plants. Drugs and their metabolites which have reached the soil are either mineralised by soil organisms or enter groundwater unaltered (Halling-Sørensen et al., 1998).

The lifetime of drugs in the environment depends on the structure of their molecules. The microorganisms of soil decompose drugs into either organic metabolites or carbon dioxide and water. The ability to produce antibiotics is a long-term evolutionary process and represents an important factor in the struggle for existence (Tshervjakova & Terezova, 1986). At the same time, the pathways of biodegradation have evolved in nature to mineralise natural antibiotics.

Synthetic and semi-synthetic antibacterial substances are currently in wide use. They are 'strangers' to nature and hard to degrade. Fluoroquinolones belong to a group of drugs that remain in the environment for a long period of time. The strong adsorption of fluoroquinolones to manure and soil can be one reason for their slow degradation (Marengo et al., 1997). The presence of enro- or ciprofloxacin in manure or soil fertilised with manure has not been studied. It is known that the elimination of enrofloxacin from animal organisms occurs through the kidneys (80%) (Crumplin, 1986). The concentration of fluoroquinolones in urine can exceed its concentration in serum up to 100–300 times (Montay et al., 1984). Enrofloxacin metabolises in animal organisms partly into ciprofloxacin (Mengozi et al., 1996). It can be concluded that arable land fertilised with liquid manure may be contaminated with fluoroquinolones (Nowara et al., 1997). The antimicrobial ability of enrofloxacin becomes stronger when ciprofloxacin is present due to synergism (Mengozi et al., 1996).

Attempts have been made to predict the concentrations of drug residues using

calculations (predicted environmental concentration – PEC). The formation of metabolites, methods of excretion, the collection and preservation of manure and the way it is spread in the field, etc. are taken into account. When animals are treated on pastures, drug residue in their excrement goes directly into the soil. In this case, the factors concerning drug residue concentration that are taken into account when making predictions are very importance. Major amounts of drug residue enter the soil with manure from animal barns.

The possible mobility of drug residue from soil to plants has been studied relatively little, although there is data on the accumulation of sulfadimetoxin in barley (Brambilla et al., 1996). The content of sulfadimetoxin was approximately four times higher in the roots than in the leaves and stems: 79 and 18 µg/g respectively (the content of sulfadimetoxin in the soil being 100 µg/g). The study concluded that a MRL (maximum residue limit) on veterinary medicine residue in plants should be imposed (Brambilla et al., 1996).

Fluoroquinolones are synthetic, wide-range drugs. Generally, the same drugs are not used on animals and humans so as to avoid the development of cross-resistance. Although the two fluoroquinolones – enrofloxacin and ciprofloxacin – are very close in structure, the former is used in veterinary medicine and the latter in human medicine. As final products of metabolism, both enrofloxacin and its metabolite ciprofloxacin end up in excrement (Boxall et al., 2006). Livestock manure is commonly used as organic fertiliser. One of its uses is on the fields where food plants are grown. The manure includes the residue of fluoroquinolones in addition to other drug residue. Plants can also intake fluoroquinolones along with minerals. The intake of drugs in small amounts can lead to drug resistance in pathogenic microbes and cause allergies and liver damage. Raw materials of animal origin are subject to strict state controls. There are no limits concerning raw materials of plant origin. The total MRL of enrofloxacin and ciprofloxacin in meat is 100 µg/kg (State Herald, 2000). In the case of raw materials of plant origin, MRL is set only for pesticide residue.

The aims of this work are to evaluate the uptake and accumulation of fluoroquinolones – enrofloxacin and ciprofloxacin – from soil into plants using a multiple concentration test and to determine their stability in soil.

## MATERIALS AND METHODS

The following materials and chemicals were used: *B. subtilis* BGA spore suspension from Merck; test agar pH 8 from Merck; trimethoprim (TMP) from Sigma; ciprofloxacin and enrofloxacin from Bayer; Petri dishes Ø 90 mm; bottomless metal cylinders Ø 6 mm; blank paper disk Ø 6 mm from Becton-Dickinson & Co; and soil free of fertilisers, pH 6.0.

The plants were grown in a room specially prepared for this purpose, aired and lighted with daylight lamps. Three plants were chosen for the experiment: lettuce (*Lactuca sativa*), common barley (*Hordeum vulgare* L.) and cucumber (*Cucumis sativus* L.). The seeds were planted in plastic pots with 1 kg of fertiliser-free soil and soil mixed with fluoroquinolone solution. The weighed soil was first mixed with fluoroquinolone solution. The necessary amount of enro- or ciprofloxacin was dissolved in 200 ml of distilled water and added to the soil. The total concentration of

the antibiotic in the pots was 10, 50, 200 or 500 mg/kg. Three parallel pots were prepared for each level of concentration. Each plant species was grown in fluoroquinolone-free soil for test purposes. The irrigation of the plants took place through a plastic tube at the bottom of the pot. This type of irrigation was necessary to avoid flushing fluoroquinolones from the upper to the lower levels and to maintain as even a concentration as possible in the pot. The experiment lasted for 28 days (42 days in the case of the lettuce). The plants were then harvested and their roots removed, dried and ground. Soil samples were taken on the 2<sup>nd</sup>, 14<sup>th</sup> and 28<sup>th</sup> days of the experiment. The soil was sterilised in an autoclave for 30 minutes under 1 bar of pressure.

In order to estimate the level of fluoroquinolones in the test soils, their adhesion level to soil particles was determined. Spiking solutions with concentrations of 0.5, 1, 2, 5, 10, 20, 40 and 80 µg/ml were prepared and mixed with test soil free of antibiotics. In order to prepare the soil samples for calibration, 2 g of air-dry drug-free soil was mixed with 10 ml of fluoroquinolone solution in 50 ml plastic centrifuge tubes using the end-over-end method over 5 hours at room temperature. The tubes were centrifuged at 4000 rpm for 30 minutes. Supernatants were removed by decantation. Sediment was dried on plastic Petri dishes at room temperature overnight. Solutions of fluoroquinolones with concentrations of 0.5, 1, 2, 5, 10, 20, 40 and 80 µg/ml were used to prepare calibration soils with concentrations of 2.5, 5, 10, 25, 50, 100, 200 and 400 µg/g respectively.

To determine the content of fluoroquinolones in the plants, their adsorption to plant material *in vitro* was estimated (Lillenberg et al., 2003). In order to prepare the calibration plants, 50 mg of dried and ground leaves and stems of three different plants grown in fluoroquinolone-free soil were mixed with 1 ml of the following antibiotic solutions in water: 0.5, 1, 2, 5 and 10 µg/ml in 2 ml Eppendorf tubes. The tubes were rotated end-over-end over 3 hours at room temperature and centrifuged at 4000 rpm for 10 minutes. Supernatants were removed using a plastic Pasteur pipette into 1 ml tubes. Sediment was dried on plastic Petri dishes at room temperature overnight.

The soil, plants, spiking solutions and supernatants were analysed using the agar diffusion method (Lillenberg et al., 2003). Three parallel determinations were performed for each sample. The measurement uncertainty of the drug concentrations was within ±10%. The test agar was sterilised in an autoclave for 50 min under 1 bar of pressure. After decreasing the temperature of the test agar to 48°C, 100 µl of aqueous solution of TMP (conc. 100 µg/ml) and 1 ml *B. subtilis* spore suspension were added to 100 ml of the medium. Petri dishes were filled with 6 ml of inoculated test agar and after a period of 30 min a Ø 6 mm stainless steel cylinder was placed on the gel. 2.5 mg of dried and ground plant material or 5 mg of autoclaved soil was poured on the gel through the cylinder, after which the cylinder was removed. In the case of antibiotic solutions or spiking supernatants, a 6 mm blank paper disk was placed on the gel and 13.6 µl of the solution was dipped on the disk. The experimental plant and soil samples and spiked samples were tested on the same gel. Petri dishes with samples on the gel were kept in a refrigerator at 4–6 °C for 22 hours for pre-diffusion, and thereafter incubated for 18 hours at 37 °C. An inhibition circle of microbial growth appeared on the gel around the sample. Its diameter was measured and the average of parallel samples calculated. The average diameters of the spiked soils were used to construct the calibration curves in axes: log from antibiotic concentrations (x) and diameter of

inhibition circles (y) of spiked soils.

The adsorption level of fluoroquinolones to the plant or soil material was determined: the spiking solutions and supernatants were analysed using the microbial inhibition test. The calibration curve was constructed in axes: log from concentrations (x) and diameter of inhibition circles (y) of spiking solutions. The concentration of the antibiotic in the supernatant showed the adsorption rate. When the supernatant produced an inhibition circle, the concentration of antibiotics in the supernatant was determined by the calibration curve. The level of adsorption of the antibiotic to plant or soil material and concentration of the antibiotic in spiked plants or soils were calculated. When the supernatant did not produce an inhibition circle, all of the added antibiotic was adsorbed by the plant or soil – an adsorption rate of 100%. The calibration curves were constructed in axis: log from calculated concentration of antibiotic in spiked material (x) and diameter of inhibition circle of spiked material.

The antibiotic concentrations in the soil and plant samples were calculated using the following equation:  $y = ax + b$ , where a and b are constants, x = log from concentration of antibiotic in sample and y = diameter of inhibition circle of sample.

## RESULTS AND DISCUSSION

### Fluoroquinolones in soil

The adsorption rate of enrofloxacin for soil at concentrations of 10, 20, 40 and 80 µg/ml was 100, 99.6, 99.5 and 99.5% respectively. These results accord with those previously published (Nowara et al., 1997), where the adsorption of enrofloxacin in soil with a pH of 5.9 was 99.5%. In the case of ciprofloxacin, the level of adsorption to soil at concentrations of 10, 20, 40 and 80 µg/ml was 100, 100, 99.9 and 99.6% respectively. The lower the level of concentration of the fluoroquinolone spiking solution, the higher the adsorption rate with soil. Generally, it can be concluded that the adsorption rate was close to 100% at all concentrations.

Table 1 and 2 show that the concentration of fluoroquinolones did not change during the experiment; this was also shown in earlier publications (Golet et al., 2002). When comparing enrofloxacin content in test soils on days 2 and 28, it can be said that no sufficient changes in the relevant concentrations could be seen (Table 1). The content of ciprofloxacin in the sample soils remained almost unchanged (Table 2).

### Fluoroquinolones in plants

The level of fluoroquinolone adsorption depended on plant species and spiking solution concentration. As the results in the soil showed, the lower the level of concentration of the fluoroquinolone spiking solution, the higher its adsorption rate. The adsorption rate of enrofloxacin in the lettuce was 64–100%, barley 28–100% and cucumber 23–100%. The adsorption rate of ciprofloxacin in the lettuce was 90–100%, barley 64–100% and cucumber 70–100%. Thereafter, the mobility of enrofloxacin and ciprofloxacin from soil to plants was studied. The results of the experiment showed that fluoroquinolones reach plants and accumulate there while retaining their antimicrobial activity. At the same initial concentration in soil, the content of

**Table 1.** Concentration of enrofloxacin in soil on 2<sup>nd</sup>, 14<sup>th</sup> and 28<sup>th</sup> days of experiment.

Plant	<i>Enrofloxacin</i> (µg/g)			
	initial	2 <sup>nd</sup> day	14 <sup>th</sup> day	28 <sup>th</sup> day
Barley	500	487	487	520
	200	193	206	206
	50	48	55	59
	10	11	13	14
Lettuce	500	455	520	555
	200	206	268	268
	50	57	65	63
	10	9	10	10
Cucumber	500	503	520	503
	200	235	220	235
	50	45	55	52
	10	10	10	11

**Table 2.** Concentration of ciprofloxacin in soil on 2<sup>nd</sup>, 14<sup>th</sup> and 28<sup>th</sup> days of experiment.

Plant	<i>Ciprofloxacin</i> (µg/g)			
	initial	2 <sup>nd</sup> day	14 <sup>th</sup> day	28 <sup>th</sup> day
Barley	500	567	494	477
	200	266	248	231
	50	62	62	54
	10	10	10	10
Lettuce	500	548	529	529
	200	284	266	248
	50	69	62	58
	10	11	11	10
Cucumber	500	629	567	529
	200	248	248	231
	50	62	54	47
	10	11	10	10

enrofloxacin was higher in the test plants than the content of ciprofloxacin. The accumulation of fluoroquinolones was dependant on plant species: its content was highest in lettuce and lowest in cucumber. The high rate of ciprofloxacin in lettuce was due to its vegetation period being 14 days longer compared to the growing period of the other plants in soil mixed with ciprofloxacin (Table 3). It is remarkable that for the lettuce grown in soil with a nominal concentration of ciprofloxacin of 10 µg/g, the content of ciprofloxacin was more than four times higher than predicted. The results showed that when the vegetation period is longer, antibiotics accumulate in the plant.

The results of the experiment showed that during the vegetation period a remarkable amount of ciprofloxacin can accumulate in lettuce. The total amount of enrofloxacin and its metabolites PEC in soil fertilised with manure could be up to 3.8

µg/g in the worst case (Halling-Sørensen et al., 2002). In our experiment with ciprofloxacin concentration of 10 µg/g in the soil, the ciprofloxacin content of the lettuce was 44 µg/g – more than four times higher in the plant than in the soil.

**Table 3.** Concentration of fluoroquinolones in plants.

Plant	<i>Enrofloxacin</i> or <i>ciprofloxacin</i> concentration in soil, µg/g	<i>Enrofloxacin</i> concentration in plant, µg/g	<i>Ciprofloxacin</i> concentration in plant, µg/g
Barley	500	20	13
	200	14	n.d.
	50	8	n.d.
	10	n.d.	n.d.
Lettuce	500	76	223 *
	200	27	163 *
	50	7	58 *
	10	n.d.	44 *
Cucumber	500	36	40
	200	22	n.d.
	50	9	n.d.
	10	n.d.	n.d.

\*the lettuce was grown for 42 days in soil containing ciprofloxacin and for 28 days in soil containing enrofloxacin

## CONCLUSIONS

Enrofloxacin and its metabolite ciprofloxacin as terrestrial contaminants must be monitored due to their wide presence in the environment, their chemical resistance and their ability to migrate from soil to crop. Enrofloxacin and ciprofloxacin uptake from soil by food plants was evident in our experiments. The significance of the route involving the uptake of the studied medicines from the soil by plants in terms of risk to human health was confirmed by our experiments. Further studies concerning the plant uptake of a wide spectrum of commonly used pharmaceuticals from soils fertilised with sewage sludge or its compost are needed with the aim of ensuring food safety.

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