Binding of Fluoroquinolone Carboxylic Acid Derivatives to Clay Minerals

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Large quantities of fluoroquinolone carboxylic acid (FQCA) derivatives are applied as antibacterial agents in large-scale animal husbandry. Important quantities are transported to agricultural areas by means of liquid manure. The binding of FQCA derivatives to clay minerals and their sorption by five soils from different geographic areas were investigated. Sorption was studied in batch experiments using radioactive labeled enrofloxacin (Baytril), decarboxylated enrofloxacin, ciprofloxacin (Cyprobay), levofloxacin (Oxaldin), and a fluorochloroquinolone carboxylic acid derivative. More than 90% (K_D = 260–5612) of the applied enrofloxacin adsorbed on the different soils. The other chemicals showed a similar adsorption (K_D = 285–496) on a German soil except the decarboxylated enrofloxacin (K_D = 7.7). At clay minerals enrofloxacin was adsorbed >98%. X-ray diffraction analysis showed that the adsorption at the clay mineral montmorillonite occurred between the layers, resulting in an expansion of the spacing. Microcalorimetric and infrared measurements confirmed an electrostatic interaction between the adsorbed chemical and the adsorbent.

Keywords: *Adsorption; clay mineral; enrofloxacin; fluoroquinolone carboxylic acid derivatives; Coulombic interactions; soil clays*

INTRODUCTION

Enrofloxacin (Baytril) is a veterinary chemotherapeutic agent with antibacterial activity. It belongs to the group of fluoroquinolone carboxylic acid (FQCA) derivatives. The mechanism of action is the impairing of the bacterial enzyme gyrase (Scheer, 1987), playing a major role in the replication of DNA. Inhibition of the gyrase leads to functional disturbances with blockage of certain sections in the DNA synthesis resulting in the death of the bacterium (Crumplin, 1986). Today, enrofloxacin is one of the most important antibacterial chemicals against Gram-negative and Gram-positive bacteria and inhibits most strains of *Mycobacterium avum*–*Mycobacterium intracellulare*. The elimination of enrofloxacin in the target organism occurs mainly as parent compound and its main metabolite ciprofloxacin (N_4-de-ethylated derivative) via the kidney (80%) and to some extent via the bile (20%) (Crumplin, 1986). Consequently, the agriculturally used soil can be contaminated by liquid manure. Little information is available on the fate characteristics of the quinolone derivatives (Yoshida, 1993; Burhenne et al., 1997). Apart from the biotic and abiotic degradation processes, the distribution and accumulation mechanisms of FQCA in soil are of interest with special reference to an ecological risk assessment.

The adsorption of xenobiotics to soil components primarily occurs on clay minerals and humic substances (Schwarzenbach et al., 1993). The layer lattice structure of phyllosilicate clay minerals offers several possible adsorption mechanisms for organics entering the soil systems (DFG, 1976). In addition to phyllosilicates, oxide, sulfide, carbonate, and phosphate clay minerals include the ability to bind organic compounds. They can be sorbed within the interlayer spaces of 2:1-type

minerals. This sorption retards their biotic and abiotic degradation. In case of chemicals sorbed in the interlayer of clay minerals, a stronger binding was expected, e.g. for phenol derivatives reported by Jackson (1993) and Klumpp et al. (1992).

To investigate the binding mechanism of FQCA derivatives, sorption investigations of enrofloxacin on five soils of different geographic origin and four different clay minerals were made. The nature and intensity of binding were determined by spectroscopic (X-ray diffraction analysis, infrared spectroscopy) and microcalorimetric (titration calorimeter) methods.

In addition to the degradation mechanisms and their structural analyses, the examinations of the sorption behavior of FQCA derivatives in the mineral soil compartment presented in this paper should lead to a further environmental risk assessment.

MATERIALS AND METHODS

Soils and Clay Minerals. Five soils of different geographic origin with varied quantities of clay and organic carbon representing a broad spectrum of different soil types from important cultivation areas (Simon et al., 1992) were chosen (Table 1). Pure clay minerals used were the nonexpanding three-layer mineral illite, the expanding minerals montmorillonite and vermiculite, and the two-layer mineral kaolinite (supplied by Messrs. Krantz, Wiesbaden, Germany).

Chemicals (Figure 1): ciprofloxacin [1-cyclopropyl-7-(1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid], pK_a = 6.38, water solubility (20 °C) = 30 g/L; enrofloxacin [1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid], pK_a = 6.27, water solubility (20 °C) = 130 g/L; levofloxacin [9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid], pK_a = 6.24; fluorochloroquinoline carboxylic acid (1-cyclopropyl-7-chloro-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid), pK_a = 6.12 (FCQCA); decarboxylated enrofloxacin [1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quin-

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Table 1. Soil Properties

country	sand (%)	silt (%)	clay (%)	H_2O^a (%)	C_{org}	CEC^b (mequiv/100 g)	pH	soil type	dominant clay mineral	clay fraction (%)
Brazil	27.2	28.8	41.7	23.1	1.63	13.6	4.9	Rhodic Ferralsol	kaolinite	22
Philippines	39.4	42.9	17.2	24.9	0.73	16.9	5.3	Glegic Cambisol	montmorillonite	21
Sweden	84.0	8.4	7.2	11.7	1.23	7.4	6.0	Haplic Podsol	montmorillonite	25
France	39.1	36.6	23.4	22.8	1.58	19.1	7.5	Rendzic Leptosol	kaolinite	8
Germany	80.1	16.7	2.5	18.3	0.70	12.0	5.3	Centric Flurisol	montmorillonite	8

^{a} 33 kPa subpressure. ^{b} CEC, cation exchange capacity.
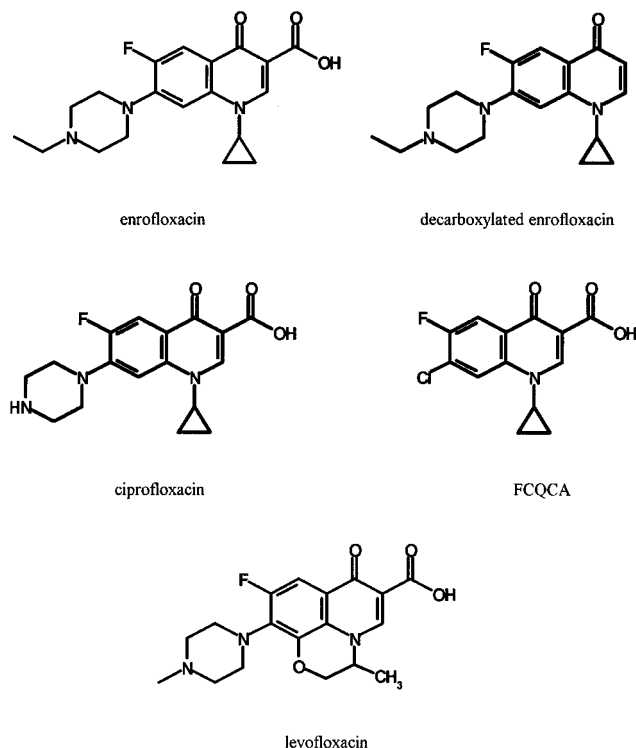
Figure 1. Structures of FQCA derivatives used.

oline] and [*carbonyl*-14C]enrofloxacin (specific activity 4.97 MBq/mg) were obtained from Bayer AG, Leverkusen, Germany.

Soil Treatment. To prevent a decline of the biological activity of the soils, they were stored with Welsh ray grass in Mitscherlich containers at ambient temperature in a greenhouse with a weekly irrigation. Before the commencement of the experiment, the soils were sieved (<2 mm) and dried for 48 h at room temperature. All weight portions refer to air-dried soil.

Clay Fractions. Isolation of clay fractions was done by a combined sieve and sedimentation method. Soil samples (20 g) were treated for 24 h with 30% H_2O_2. Thereafter the soil was dispersed in 50 mL of 0.2 M $Na_4P_2O_7$. The isolation of the coarse fractions (2–0.06 mm) was obtained by using three sieves of different mesh sizes. The determination of particle sizes 0.06–0.002 mm and the isolation of clay fraction (<2 μm) were carried out according to the pipetting method (Scheffer and Schachtschabel, 1992). After a sedimentation period of 212 min, 20 mL aliquots of the soil suspension were taken (depth 10 cm) and dried (40 °C).

Homoionic Saturation. Since clay samples were analyzed, it was advisable to exchange and saturate the clay with a cation minimizing changes in interlayer water adsorption due to fluctuations in relative humidity. Magnesium (Mg^{2+}), which allows uniform interlayer adsorption of water by expansible phyllosilicates, and potassium (K^+), which restricts interlayer adsorption of water, were used for the exchange and saturation (Walker, 1957). For Mg^{2+} saturation a suspension of 250 mg of clay fraction with 2 mL of Millipore H_2O, equilibrated to pH 4.5 with 0.1 M HCl, and 5 mL of Mg(OAc)₂ (5 M) were added, and the suspension was shaken on a rotary shaker for 10 min (30 rpm). After centrifugation (1500 rpm,

10 min) and decantation, 5 mL of 5 M Mg(OAc)₂ was added, followed by three treatments with 20 mL of 0.5 M MgCl₂. The Mg^{2+}-saturated samples were washed with H_2O, methanol, deionized water, and acetone until all salts were removed. Finally, the clay fractions were dried at 40 °C. The K^+ exchange was achieved by application of 20 mL of 1 M aqueous KCl solution to 250 mg of clay suspension. After centrifugation (1500 rpm, 10 min) and decantation, the clay was mixed four times with 20 mL of 1 M KCl solution, stirred, siphoned off, and dried at 40 °C. The K^+-saturated samples were treated in a manner similar to the one used for Mg^{2+}-saturated samples.

Adsorption/Desorption. The adsorption isotherms were obtained using the batch method with radioactive labeled enrofloxacin. Substrate solution (20 mL, concentration 0.1–10 mg/L) in PTFE centrifugation tubes were added to 1.00 g of soil or clay mineral and shaken for 24 h (dark, 20 ± 1 °C; 30 ± 1 rpm), although the equilibrium was reached after 6 h. The samples were centrifuged (8000 rpm, 20 min, 10 °C), the solid phase was separated, and the liquid phase was analyzed by liquid scintillation counting (LSC) and HPLC. pH measurements of substrate solutions and clay suspensions as well as of reacting solutions were done with a pH-meter (Orion, Model 720).

The desorption of enrofloxacin was carried out by 0.01 M CaCl₂, methanol, and 0.1 M tetrabutylammonium hydroxide (pH 12.5), 20 mL each. After 24 h of incubation (equilibrium reached after 6 h again), the radioactivity in the liquid phase was measured by LSC. The radiochemical purity of [14C]-enrofloxacin was verified by HPLC. The concentration of nonradioactive ingredient in the supernatants after equilibrium was measured by HPLC with UV and fluorescence detection.

Radioactivity and Chromatographic Measurement. LSC (LS 6500, Messrs. Beckmann) was performed using Quicksafe A and Quicksafe B as scintillators for aqueous respectively organic solutions. Samples were measured for a period of 20 min or until the standard deviation was below 0.5%.

The solid phase adsorbed radioactivity was determined by combusting three aliquots in a Biological Material Oxidizer OX 500 (Harvey Instruments) and subsequent measurement of the evolved $^{14}CO_2$ by LSC.

HPLC measurements were performed by a Waters modular HPLC system under the following conditions: column, Alltech RP-18, 5 μm, 250 mm × 4 mm i.d.; eluent A, Milli-Q H_2O/H₃PO₄ (1 mL/L); eluent B, CH₃CN; linear gradient, 0 min/0% B–1 min/0% B–60 min/60% B; flow rate, 1 mL/min; injection, 10–50 μL; detection, UV 274 nm; fluorescence, λ_{Ex} = 274 nm λ_{Em} = 455 nm; radioactivity (type Ramona, Messrs. Raytest), flow-through-cell with liquid scintillation cocktail "Monofluor" (National Diagnostics) 2 mL/min.

Microcalorimetric Measurement. A large test tube was used as reaction vessel (Wöhrmann, 1985). To guarantee thermal insulation, the tube was surrounded with a Moltopren layer. The samples were stirred with a magnetic stirrer. Substrate solution was continuously added by a Dosimat E 535 (Metrohm Herisau). The volume and drop rate (10 mL/min) of substrate solution could be exactly controlled. The temperature was measured by a resistance thermometer (calibration leads to a 15 mV/°C sensitivity). Temperature/time curves were recorded by a wheatstone bridge and a parallelly connected voltmeter. The clay mineral exchange enthalpies were measured with a solution of 10 mL of Millipore H_2O and 1.0 g of phyllosilicate. A 300 mg enrofloxacin solution in 100 mL of Millipore H_2O (substrate concen-

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Table 2. Enrofloxacin Sorption on Soil

soil origin	adsorption					desorption				
	%	K_D	K_{OC}	K_D (clay)	pH	%[a]	$K_D{}^a$	%[b]	%[c]	% Σ
Brazil	99.7	3037	186342	330	4.8	0.2	4002	0.7	39.9	40.8
Philippines	99.7	5612	768740	2240	5.0	2.6	27504	2.4	61.4	66.4
Sweden	98.6	1230	99975	2276	5.3	0.2	3347	0.3	73.0	73.5
France	95.2	260	16506	140	6.6	0.9	687	1.2	78.1	80.2
Germany	95.5	496	70914	2480	5.0	1.3	1224	1.5	79.8	82.6

[a] 0.01 M CaCl$_2$. [b] CH$_3$OH. [c] 0.1 M tetrabutylammonium hydroxide (TBAH), pH 12.5.

tration = 8.3 mmol/L) was used as sorbate. The sensitivity of the method used was about ±4 kJ/mol (3-fold measurement).

X-ray Diffraction Analysis. Phyllosilicates were examined by diffraction from (00*l*) planes by a diffractometer PW 1050/25 (Messrs. Phillips, voltage = 40 kV, current intensity = 25 mA). A linear counter was used to intercept and measure the diffraction rays. Copper radiation (Kα, 0.154 nm) was used as the characteristic wavelength.

Clay samples (0.3−0.5 g) were equilibrated with the test compound (10−200 mg/L), separated by centrifugation, dried, and mounted on a metal wedge. *D/n* values for diagnostic basal (00*l*) diffraction maxima, Mg-saturated (air-dried), and K-saturated (air-dried) pure clay minerals as well as solid enrofloxacin and ciprofloxacin were measured additionally.

IR Measurement. Infrared spectra were obtained using a FTS 40A-spectrometer (Bio-Rad) with He−Ne laser (632.8 nm). Solids were measured as KBr tablets between 4000 and 400 cm^{-1}.

RESULTS AND DISCUSSION

Sorption. The content of clay in the different soils varies between 2.5 and 41.7% and consists mainly of montmorillonite and kaolinite. The portion of pure clay mineral in the clay fractions of the soils as well as the content of organic carbon and other parameters are given in Table 1. These soils adsorb at least 95.2% of the radioactive labeled enrofloxacin (Table 2). The substrate is nearly completely bound by the soils from Brazil and the Philippines. The large clay fractions and the huge amounts of pure clay minerals provide a high adsorption surface. Consequently, the desorption is very low. The Swedish soil shows a high adsorption and low desorption of enrofloxacin, too, because the content of pure montmorillonite in the relatively small clay fraction is about 25% (Table 1). The German and French soils adsorb the lowest amounts of enrofloxacin (about 95%), but the reasons are different. The German soil contains only 2.5% clay, in which 8% montmorillonite can be determined. Additionally, the content of organic carbon, which has a secondary influence on the sorption of enrofloxacin, is low (C$_{org}$ = 0.7%). The French soil shows a 23.4% content of clay, but the dominating clay mineral is kaolinite, with a content of only 8%. Calculations based on the adsorption of pure clay minerals show the differences in the extent of adsorption between montmorillonite and kaolinite (Table 2). Kaolinite soils (Brazil, France) show K_D values based on the clay mineral content that are considerably lower than those of montmorillonite-containing soils (Germany, Sweden, Phillippines). The isolated clay fractions of the different soils sorb a very high amount of the added substrate (Table 3). The slightly lower adsorption of the clay fraction isolated from the German soil can be explained by the low content of the clay mineral montmorillonite as mentioned above. Different desorption results from isolated and pure clay minerals (Table 4) lead to the assumption that a high amount of phyllosilicate in the clay fraction of the soils increases

Table 3. Enrofloxacin Sorption on Isolated Clay Fractions

clay fraction	adsorption		desorption			
	%	pH	%[a]	%[b]	%[c]	Σ
Brazil	99.6	5.3	0.4	0.9	40.3	41.6
Philippines	99.6	5.8	0.4	0.7	41.8	42.9
Sweden	99.5	5.9	0.5	1.0	43.1	44.6
France	99.2	6.8	1.0	1.0	54.7	56.7
Germany	98.7	5.7	1.8	1.7	59.6	63.1

[a] 0.01 M CaCl$_2$. [b] CH$_3$OH. [c] 0.1 M tetrabutylammonium hydroxide (TBAH), pH 12.5.

Table 4. Enrofloxacin Sorption on Pure Clay Minerals

clay mineral	adsorption			desorption			
	%	K_D	pH	%[a]	%[b]	%[c]	Σ
kaolinite	98.9	3548	6.0	0.2	0.4	49.3	49.9
illite	99.3	4670	6.1	0.2	0.3	46.6	47.1
vermiculite	99.7	5986	5.8	0.2	0.2	41.6	42.0
montmorillonite	99.8	6310	5.9	0.1	0.2	32.1	32.4

[a] 0.01 M CaCl$_2$. [b] CH$_3$OH. [c] 0.1 M tetrabutylammonium hydroxide (TBAH), pH 12.5.

Table 5. Sorption of Quinolone Derivatives on Soil (Germany)

quinoline derivative	adsorption	
	K_D	K_{OC}
enrofloxacin	496.0	70914
decarboxylated enrofloxacin	7.7	1100
ciprofloxacin	427.0	61000
ofloxacin	309.0	44143
FCQCA	285.0	40714

the adsorbability and decreases the desorption. The clay fractions of German and French soils contain only a low percentage of montmorillonite (8%), explaining the relatively high desorbability. The other clay fractions contain pure minerals up to 21%, which explains the reason for the low desorbability of quinolone derivatives. The organic compound can be sorbed within the interlayer spaces of 2:1-type minerals (Hower, 1970), on broken-bond surface, and on the basal oxygen and hydroxyl planes. Kaolinite and illite sorb only on basal surfaces and edges. Vermiculite clay minerals are known to expand up to 1.42 nm and therefore show an adsorption similar to montmorillonite.

Sorption measurements with different fluoroquinolones at one soil result in similar K_D values for the substances containing a carboxylic group (Table 5). The different substituents in the quinolone structure (piperazine ring, chlorine, etc.) have no essential influence on the extent of sorption. The fundamental structural element leading to a high adsorption of fluoroquinolones seems to be the β-keto acid structure. This element is not present in the case of decarboxylated enrofloxacin, and therefore this compound is adsorbed to a significantly lower extent (K_D = 7.7).

Due to the pH values measured in the soil solutions and clay suspensions (Tables 2−4) equilibrated with the
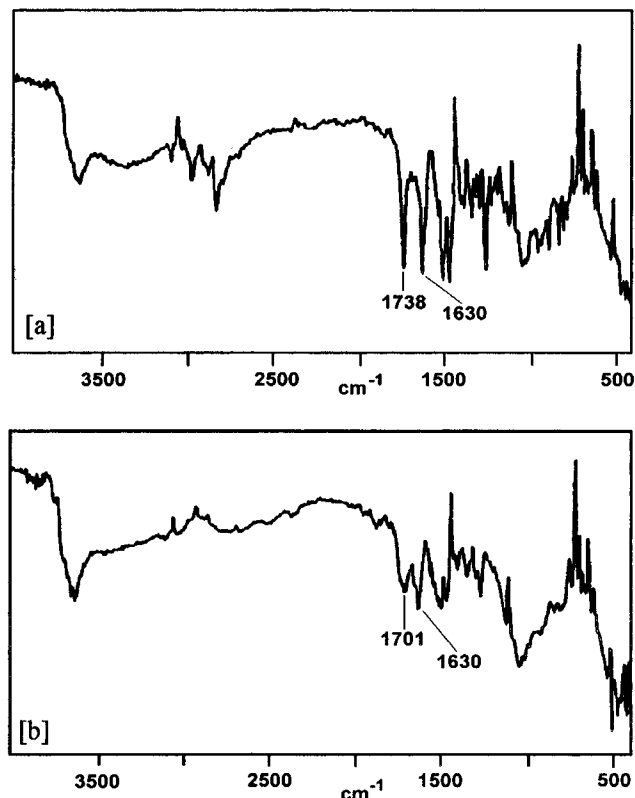
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Figure 2. IR spectra: (a) mechanical mixture; (b) adsorbed enrofloxacin.
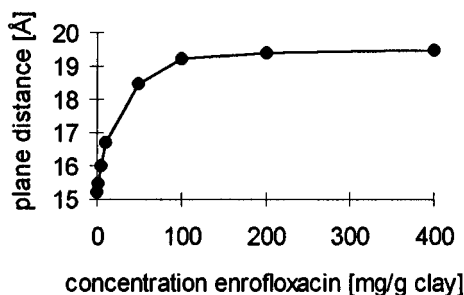
Figure 3. Montmorillonite spacing as affected by enrofloxacin sorption.
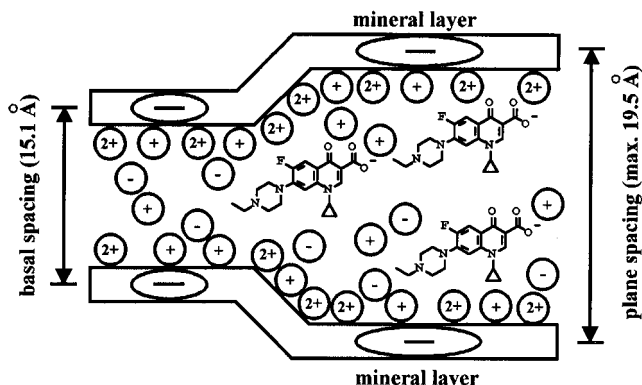

Figure 4. Binding of FQCA derivatives on clay minerals.

Table 6. Microcalorimetric Measured Exchange Enthalpies

clay mineral	mass (g/unit cell)	ciprofloxacin (kJ/mol)	phenol (kJ/mol)	KCl (kJ/mol)
kaolinite	282	19.5	18.9	12.8
illite	374	32.0	27.8	20.7
vermiculite	374	39.8	35.9	29.1
montmorillonite	385	42.2	36.9	31.1

weak acid enrofloxacin (pK_a = 6.27), the degree of dissociation can be calculated to about 10%. This amount of ionized ingredient is able to interact with cations on the basal surfaces as well as with cations in the interlamellar space of expandable clay minerals. This sorption leads to a further dissociation of enrofloxacin to maintain the equilibrium. The high capacity of the soil and clay minerals is able to adsorb the applied compound ionically. Along this process the abstracted protons can be buffered by the soil and do not influence the pH of the soil solutions significantly.

FTIR Investigations. The mechanical mixture of montmorillonite and enrofloxacin shows an overlapping of the IR signals of pure ingredient and pure clay mineral (Figure 2a). The vibration of the OH function at 3600 cm^{-1} and less specific vibrations at <1500 cm^{-1} (Al−O, Al−O−Si, O−Si) also appear in the spectra of pure montmorillonite. In the enrofloxacin spectra aromatic CH vibrations at >3000 cm^{-1}, skeleton vibrations at 1500 cm^{-1}, and deformation vibrations (about 1400 cm^{-1}) can be observed. The IR spectrum of sorbed enrofloxacin (Figure 2b) describes a clear alteration of the carboxylic group from 1738 cm^{-1} (mechanical mixture) to 1701 cm^{-1}. It can be assumed that the sorption

of enrofloxacin proceeds via the ionized carboxylic group. This is supported by titration experiments of montmorillonite with enrofloxacin. Increasing amounts of enrofloxacin cause a reduction of the initial pH in solution. In the two spectra the quinolone keto function remains unchanged (1630 cm^{-1}). Therefore, a modification of the quinolone ring in adsorbed enrofloxacin is unlikely. The unchanged appearance of other enrofloxacin-containing signals, also in the spectra of the adsorbed substrate, excludes other modifications caused by binding at the quinolone skeleton, the piperazine ring, or other substituents.

Microcalorimetry. The microcalorimetric measurements confirm the dependence of available mineral surface and adsorbed substrate. The amount of evolved heat increases with the accessible mineral surface for the substrate (Rheinländer, 1993) (Table 4). Because of the relatively low exchange enthalpies measured (13−43 kJ/Mol), covalent bonds (>300 kJ/Mol) between clay mineral and enrofloxacin can be excluded. However, these enthalpies allow the conlcusion that a dipole or ionic binding is probable. These results correspond to those obtained by IR spectroscopic investigations from Ziechmann and Müller-Wegener (1990), who reported similar exchange enthalpies for phenol (Table 6). Phenol (pK_a = 10) reacts in water as proton donator and is fixed ionically at the mineral surfaces.

X-ray Diffraction Analysis. In contrast to other clay minerals, the 2:1 layer lattice phyllosilicate is able to sorb organic molecules within the interlayer spaces. Investigations at K$^+$- and Mg^{2+}-saturated and air-dried montmorillonite show interlayer spacings of 1.08 nm (K$^+$) and 1.52 nm (Mg^{2+}). The sorption results of enrofloxacin at Mg montmorillonite prove the storage of enrofloxacin between the mineral layers. The spacing increases with the degree of concentration of enrofloxacin (Figure 3). Saturation with substrate results in a maximum interlayer expansion of 0.44 nm. The maximum spacing is reached at a concentration of 100 mg of enrofloxacin/g of clay.

Taking the adsorption data of the Philippine soil clay fraction containing montmorillonite leads to the same results. They also show an increase in basal spacing depending on the applied amount of enrofloxacin. The

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storage of enrofloxacin between the layers of expanding phyllosilicates can cause an accumulation in soil and consequently reduces the possible desorbability. Other FQCA derivatives such as ofloxacin show a similar behavior.

CONCLUSION

On the basis of the results obtained, a binding model of ionized FQCA derivatives with phyllosilicates is proposed. The quinolone acid derivatives appear in soil solution partly as anions and interact with the exchangeable cations which are bound to the negatively charged mineral surfaces (Stern layer). Dissociated enrofloxacin is fixed (cation bridge) to divalent and monovalent cations in the diffuse interlamellar space. There are only ionic interactions between the reaction partners (exchange enthalpies <43 kJ/mol). In absence of the β-keto acid structure of the quinolone derivatives the sorption is reduced by 2 orders of magnitude.

In the case of phyllosilicates, especially montmorillonite, the sorption of the substrate leads to an increase in the interlayer distance (maximum spacing 0.44 nm). It can be assumed that a maximum of the substrate will be sorbed when the molecules are orientated as flatly as possible, which is favored by the plane structure of the FQCA derivatives. Disordered or perpendicular arrangement of the substrates leads to a lower adsorption capacity.

Soils with a variety of different pedological parameters readily adsorb the FQCA derivatives nearly 100%, and the desorption is low. This behavior is attributed to the clay mineral's ability to preferably adsorb plane anionic substrates between the mineral layers and at the outer surfaces via Coulombic interactions.

Further experiments on the bioavailability of adsorbed quinolone carboxylic acid residues for soil organisms and plants as well as their long-term fate in soil are necessary for an environmental risk assessment. The results presented in this paper show that the high sorption of FQCA to different soils prevents leaching into ground or surface waters.

ACKNOWLEDGMENT

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