Oxytetracycline Assay in Pond Sediment

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Abstract

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The fate of drug residues and their metabolites in the environment is relatively rarely investigated in the conditions of the Czech Republic, resulting in limited availability of scientific information. To demonstrate one example, we prepared a model study with medicated feedstuff containing oxytetracycline hydrochloride (OTC HCl), which was used in fish under normal conditions of use. The oxytetracycline (OTC) contents were determined in the sediments of the pond where the fish were treated. The ELISA method was used for OTC detection and the HPLC method was used for final quantification of OTC. The increasing contents of OTC in sediment depended on the repeated treatment and excretion of OTC by the fish. The concentration on Day 59 after the last administration was 1516 $\mu g \cdot kg^{-1}$ OTC in the sediment, which indicates a long-term persistence of the substance in the environment.

Antibiotics, HPLC, ELISA, medicated feeding stuff, fish

The safety of veterinary pharmaceuticals needs to be monitored not only with respect to the target animal species and humans, but also regarding the environmental contamination. One of the most frequently used groups of pharmaceuticals is antibiotics. Of about 13,500 tonnes of antibiotics administered in Europe every year, about 35% are used in veterinary medicine (Christensen et al. 2006). In fish farming, where 70 to 80% of antibiotics are administered in medicated pelleted feedstuffs, substances are released to the aquatic environment from urine, faeces and also uneaten feed (Hektoen et al. 1995). In their more detailed list of the most important substances, Boxall et al. (2003) included amoxicillin, dihydrostreptomycin, enrofloxacin, lincomycin, oxytetracycline, sulfadiazine and tylosin. Other groups of pharmaceuticals that need to be regarded include endectocides, coccidiostatics, antiprotozoics, antimycotics, hormones, antibiotic growth enhancers, anaesthetics, tranquilizers, non-steroid antiphlogistics, etc.

One of the most direct routes is drug administration (and antibiotics in particular) to fish where they are used for mass medication purposes in medicated feeds. This application may, however, lead to accumulation of excreted and uneaten drug residues in the aquatic environment. It results in an increase in the numbers of resistant microorganisms. That has been studied and demonstrated in samples from fish farm outlets (Schmidt et al. 2000). It is estimated that over 75% of the majority of antibiotics disseminated to water at fish rearing facilities spread to the external environment (Richardson et al. 1985; Halling-Sørenson et al. 1998).

In the Czech Republic, infectious diseases in cyprinids are treated using oxytetracycline, a broad-spectrum antibiotic that is administered as granulated medicated feed to ponds in quantities adequate to the stock size (Svobodová et al. 2006). Its action affects protein

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synthesis in microorganisms. Oxytetracycline is generally considered a very stable substance in the environment, with half-life of 101 to 364 days (Zuccato et al. 2001).

The aim of the study presented here was to determine oxytetracycline concentrations in the bottom sediment in dependence on the time following the recommended administration of the registered veterinary drug Rupin Special gran. ad us. vet. under semi-operational conditions. Another aim was to select suitable methods for the assay of oxytetracycline residues.

Materials and Methods

Materials

The demonstration experiment was performed in a pond 0.16 ha in size and stocked with K_2 carp. The fish were fed medicated feed registered in the CR for cyprinids, Rupin Special gran. ad us. vet., at a concentration of 5 g oxytetracycline hydrochloride/kg feed according to the instructions for therapeutic purposes. The feed was fed 4 times in September 2005, with two-day intervals between administrations. The daily dose of 10 kg of the medicated feed (Rupin), i.e. 15 g Rupin Special gran. ad us. vet. per 1 kg of fish stock, was placed on the permanent feeding place where the fish were accustomed to feed regularly.

Samples of the sediment were collected from the feeding place and the pond inlet. The sediment samples collection started before the beginning of treatment (Day 0) and continued until Day 12 at one-day intervals, and then the collection interval was extended to one week. The collection of samples was completed on Day 69, when the fishpond was harvested. The samples were frozen after collection and stored at -20 °C. Water temperature, pH and oxygen concentrations were monitored throughout the collection period of 70 days. The sample collection overview is given in Table 1.

Analytical methods

ELISA was used as a screening method to get an estimate of oxytetracycline concentrations in sediments, and HPLC method with UV detection was then used for the determination of sediment oxytetracycline concentrations.

ELISA was performed using a commercial testkit manufactured by R-Biopharm (Darmstadt) - Art. No. R3501, RIDASCREEN® Tetracycline.

Because the testkit was designed to detect tetracycline groups in meat, milk and honey, extraction procedures given there had to be modified for pond sediments. Another factor that had to be taken into consideration was the 10 × lower sensitivity of the testkit for OTC than for CTC, TC, minocycline and rolitetracycline. These factors had to be taken into account in the result evaluation.

The results obtained were used for basic information and identification of sediment samples with high concentrations of OTC that had to be diluted before they were used in subsequent confirmation assays. The HPLC method was used for the assay.

HPLC with detection at 360 nm is used to assay residues in biological material with the following validation parameters: detection limit of 25 $\mu g \cdot kg^{-1}$; precision level of 100 $\mu g \cdot kg^{-1}$ - RSD = 4.7%; yield level of 100 $\mu g \cdot kg^{-1}$ - 61.5%; correlation coefficient of calibration curve in the range 50–250 $\mu g \cdot kg^{-1}$ - > 0.99.

The applicability of the method was tested on a control sediment sample and a sediment sample with an admixture of a known amount of oxytetracycline (standard oxytetracycline substance, SIGMA ALDRICH Lot 69HO661).

Oxytetracycline was extracted from the mud sample using the McIlvain buffer. The liquid phase was cleaned and partly concentrated using the SPE technique on the column Sep-Pak Plus C18 Waters. Following elution with Chelatone III saturated methanol (p.a., LACHEMA), eluate was evaporated to dryness using a vacuum evaporator at a maximum water bath temperature of 55 °C. The residue on evaporation was diluted in 1 ml of mobile phase. In the case of higher values, samples were diluted. Thus obtained solutions were filtered and dosed to the chromatograph under the following conditions:

Column:	Hypersil MOS Supelco inc. (5 μm; 4.6 mm x 150 mm)
Mobile phase:	0.63 g oxalic acid p.a. (LACHEMA)
	1.08 g sodium octansulfonate (SIGMA ALDRICH)
	310 ml acetonitrile, Lichrospher (SIGMA ALDRICH)
	ad 1000 ml distilled water
Detection:	360 nm

The values obtained were calculated automatically by the data processing system of the instrument when the data were added to the calibration curve using the external standard method.

Results and Discussion

Oxytetracycline residue concentrations obtained by ELISA and values obtained by HPLC are given in Table 1.

Table 1. Overview of sampling procedures and oxytetracycline concentrations in bottom sediment

Sampling date in 2005	Days after application	Oxytetracycline concentrations [µg·kg^{-1}]	
		ELISA	HPLC
Samples (a) - feeding place			
8. 9.	0	166	83
9. 9.	1 (Rupin)	185	95
10. 9.	2	274	171
11. 9.	3	261	151
12. 9.	4 (Rupin)	140	93
13. 9.	5	388	209
14. 9.	6	462	331
15. 9.	7 (Rupin)	355	265
16. 9.	8	187	175
17. 9.	9	1211	1252
18. 9.	10 (Rupin)	1819	2075
19. 9.	11	2040	1936
20. 9.	12	958	820
27. 9.	19	1635	1531
4. 10.	26	1026	952
11. 10.	33	1192	1189
18. 10.	40	858	958
26. 10.	48	527	643
1. 11.	54	1160	1701
8. 11.	61	1216	1730
16. 11.	69	1281	1516
Samples (b) - pond inlet			
19. 9.	11	101	54
20. 9.	12	133	75
27. 9.	19	141	98

Rupin = medicated feedstuff Rupin Special gran. ad us. vet.

On Day 0, i.e. prior to the experiment, oxytetracycline sediment concentration was found to be 83 µg·kg^{-1} of sediment. Following the treatment of fish, concentrations did not show any marked increase and ranged between 95 to 331 µg·kg^{-1} sediment (Days 1 - 6). A day before the last application (Day 9), oxytetracycline concentration already reached 1252 µg·kg^{-1}. An important finding was that the OTC concentration at the feeding place almost two months after the last application was still at 1516 µg·kg^{-1} of sediment. Because no increase in OTC concentrations was found in samples from the inflow area, only three samples were analyzed by HPLC. OTC was also detected there at concentrations of 54, 75 and 98 µg·kg^{-1} sediment, respectively, and these results correspond to the level of OTC in the sediment detected before starting the treatment. Therefore, analyses of further samples from the inflow area were not performed.

Water temperature, pH and oxygen concentrations were within limits characteristic for pond water at that time of the year.

There was a correlation between ELISA and HPLC results. Comparison of the values obtained by the two methods is presented in Fig. 1. Because of the abnormal distribution of values, the non-parametric Spearman correlation coefficient was used to correlate ELISA and the HPLC results. To trace the decrease in oxytetracycline sediment concentrations, linear regression was used over dates following Day 11 after the first administration (until

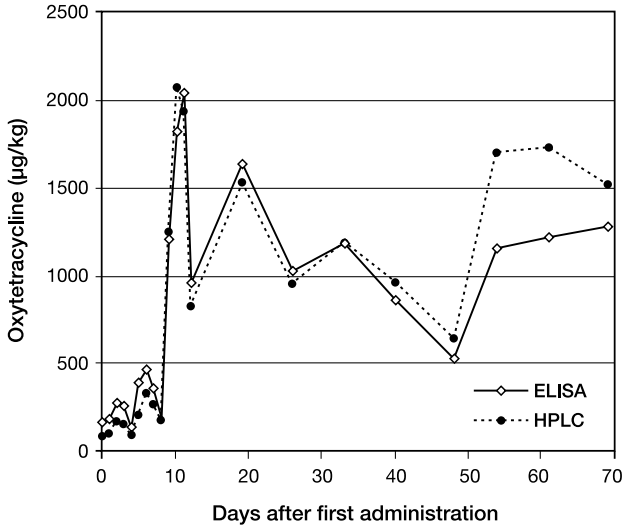

Fig. 1. Oxytetracycline concentrations in bottom sediment

incl. 19.09.). That date was selected because the last administration of feed took place on 18.09.

A significant positive correlation was found between ELISA and HPLC ($r_s = 0.969$; $P < 0.001$) (Fig. 2).

Using ELISA, no significant decrease in oxytetracycline sediment concentrations were found after oxytetracycline administration (oxytetracycline = 1463.14 - 7.34*day-after; $r^2 = 0.131$; $P = 0.305$); neither did HPLC find any significant trend in the decrease of oxytetracycline sediment concentrations (oxytetracycline = 1185.39 + 3.01*day-after; $r^2 = 0.019$; $P = 0.701$).

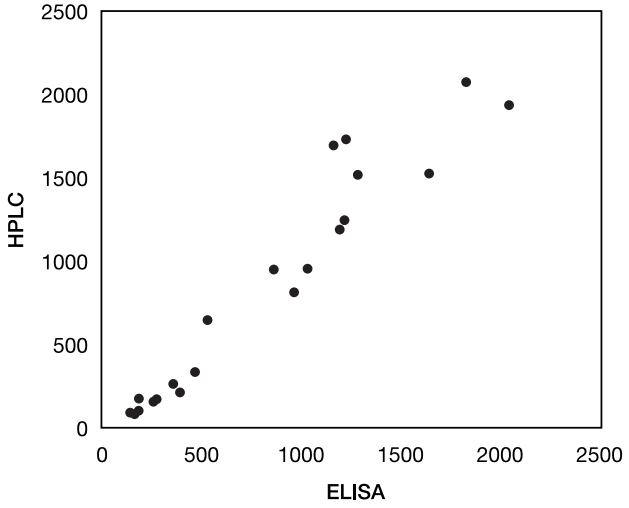

Fig. 2. Diagram of ELISA and HPLC results correlation (µg·kg^{-1} OTC in sediment)

The aim of this demonstration study was to show what OTC concentrations in sediments may be expected after standard OTC treatment administration. Because it was necessary to choose suitable methods for the assay, OTC detection sensitivity was also tested by ELISA. ELISA was used as a screening method facilitating the approximate determination of OTC concentrations in samples, which were then further analyzed by HPLC. ELISA met the requirements for screening methods, i.e. it was able to detect the analyte, or a group of analytes, within limits appropriate for the purpose, guaranteed the minimum incidence of false negative results, and made it possible to process a large number of samples in a short period of time. A significant correlation was found between the two methods ($r_s = 0.969$).

After administration, oxytetracycline is deposited in sediments where its concentrations increase not only when feed is applied to ponds, but also when OTC is excreted by fish. Our results showed that OTC concentrations remained unchanged even two months after the last use of medicated feedstuff. The concentration was 1516 $\mu g \cdot kg^{-1}$ sediment and no significant decrease in concentration was recorded. This confirms literary data (Zuccato et al. 2001), suggesting a long-term persistence of OTC in the environment. Samuelsen et al. (1992) also confirmed a long half-life of oxytetracycline (from 87 to 144 days after the end of therapy) in ocean sediments.

The accumulation of antibiotic residues in ecosystems leads to the development of resistance in microorganisms. This is also the risk involved in the therapeutic use of oxytetracycline in fish farming. Petersen et al. (2002) reported a 100 per cent resistance in *Enterococcus* spp. and *Acinetobacter* spp. isolated from pond sediment samples after a two-month accumulation of oxytetracycline residues.

Another interesting result was the finding of OTC sediment contamination (83 $\mu g \cdot kg^{-1}$ OTC) in the pond even prior to the beginning of treatment. This is suggestive of OTC use in previous years, or of possible contamination of the water source. Low oxytetracycline concentrations in sediments were also found by Capone et al. (1996) and Jacobsen and Berglind (1988). This is due to the frequent use of oxytetracycline for the treatment of fish world-wide.

Stanovení oxytetracyklinu v rybničním sedimentu

Osud reziduí léčiv a jejich metabolitů v prostředí je relativně velmi málo sledovanou oblastí, což vede k omezenému množství dostupných vědeckých informací v České republice. Cílem naší práce bylo představit jeden případ a připravit modelovou studii použití medikované krmné směsi obsahující oxytetracyklin-hydrochlorid (OTC HCl), která byla podána rybám za standardních podmínek. Sledovali jsme koncentrace oxytetracyklinu (OTC) v rybničním sedimentu takto ošetřeného rybníka. Pro detekci OTC byla využita metoda ELISA a HPLC metoda byla použita pro konečnou kvantifikaci OTC. Zvyšování koncentrace OTC závisí na opakovaném podávání a vylučování OTC rybami. Koncentrace 59. den po posledním dni aplikace dosahovala hodnoty 1516 $\mu g \cdot kg^{-1}$ OTC v sedimentu a dokládá dlouhodobé setrvávání látky v prostředí.

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