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SPE extraction and TLC Identification of Tetracycline and Fluoroquinolone in Surface Water

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Abstract: Simultaneous identification of the antibiotics tetracycline, oxytetracycline, chlortetracycline, ciprofloxacin and enrofloxacin in surface water is reported. The method is based on solid-phase extraction (SPE), separation and identification by thin-layer chromatography (TLC). TLC separation was performed on TLC silica gel 60 F254 plates using a mobile phase system water/methanol/dichloromethane (6/35/59) (v/v). The plates were previously impregnated with 10% solution EDTA pH 9,0 and dried in a horizontal position for at least two hours at room temperature and then in an oven at 105°C 30 minutes shortly before use. Antibiotics were extracted on the OASIS HLB 6cc/500 mg cartridges. Aliquots of 10 µl of the water sample and reference solutions were applied to the plate. After development the plates were air dried and the chromatograms were visualized under UV light at λ = 254 nm and λ = 366 nm. Proposed method can be applied for screening of investigated antibiotics in water samples were antibiotic concentration is equal or higher than 5 µg/ml.

INTRODUCTION

In recent years, pharmaceuticals have been detected in a wide variety of environmental samples including sewage effluent, surface waters, groundwater and drinking water at a concentration from ng/l to µg/l. They are considered as pseudo-persistent pollutants, which continually enter the environment at very low concentrations (Valverde, García, Galera *et al*., 2006).

Among different groups of pharmaceuticals, antibiotics are of special concern: large quantities are administered to humans and animals to treat diseases and infections. They are also used at sub-therapeutic levels to promote growth in livestock. After application, many of them are excreted unchanged or completely metabolized to inactive compounds, but a significant amount is excreted as active metabolites. The most prominent effect of antibiotics in the environment is the development of multi-resistant strains of bacteria (Hirsh, Hernes, Haberer *et al.* 1999, Valverde, Gil García, Galera, *et al.* 2006).

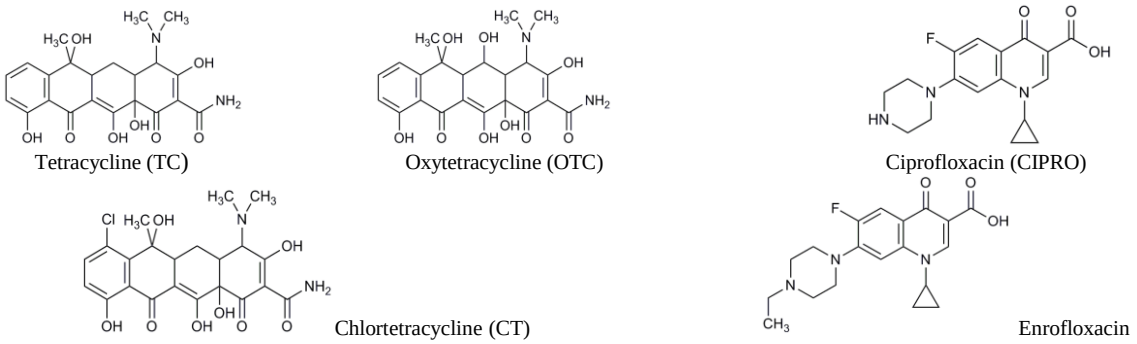
Fluoroquinolones and tetracyclines are commonly used in human and veterinary medicine and there is danger from their presence in the aquatic environment (Lindberg, Jarnheimer, Olsen *et al.,* 2005, Turiel, Bordin and Rodríguez, 2005, Brown, Kulis, Thomson *et al*., 2006). Depending on the chemical properties of the individual groups of antibiotics excretion of unchanged active compound is 10-90% (Boxal, Fogg, Kay *et al.,* 2003). Tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) are antibiotics of the tetracycline group frequently given to animal destined for human consumption, not only to prevent and treat certain diseases but also to accelerate growth (Stockwell and Duffy, 2012, Serrano, 2005). After medication, more than 70% of tetracycline antibiotics are excreted and released in environment in active form via urine and feces (Kuldip, Satish, Gupta *et al.* 2005). Fluoroquinolone residues can enter the environment mainly as a result of their excretion in the urine of humans and animals, as well as of aquaculture treatments (Boxal *et al.,* 2003, Turiel, Bordin and Rodríguez, 2005, Andreu, Blasco and Pico, 2007).

Numerous analytical methods are currently available to detect tetracyclines and fluoroquinolones in different samples including UV-Vis spectroscopy (Galagher and Danielson, 1995), fluorescence (Smirnova, Yu and Zhmerichkin, 2005), capillary electrophoresis (Miranda, Rodríguez and Galán-Vidal, 2009), high performance liquid chromatography (Blackwell, Holten Lützhøft, Hai Ping *et al.*, 2004) and liquid chromatography-tandem mass spectrometry (Schneider and Donoghue, 2002).

There are numerous literature data that describe the requirements for the identification and quantification of tetracyclines and fluoroquinolones in various samples using thin layer chromatography (Belal, Al-Majed and Al-Obaid, 1999, Thangadurai, Shukla and Anjaneyulu, 2002).

Separation, identification and quantification of tetracyclines and fluoroquinolones are possible on silica gel, cellulose and polyamide stationary phase. Most of the published methods requires impregnation of the TLC plate with EDTA solution before applying the samples on the plate in order to avoid the formation of the complex and hence improved separation (Feng and Dung, 2004, Naidong, Cachet, Roets *et al.*, 1990, Dong, Xie, Shuang *et al*., 1999). The aim of this study was preliminary testing of the presence of residues of tetracycline, oxytetracycline, chlortetracycline, enrofloxacine and ciprofloxacine in water samples collected from two defined localities.

Figure 1: Chemical structure of tested substances



MATERIALS AND METHODS

Acetonitrile was HPLC grade (Panreac, Italy) and the other used solvents were of p.a. grade (Merk, Germany). Tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CT), enrofloxacin (ENRO), ciprofloxacin (CIPRO) were min. 98% pure (Figure 1). Stock solutions of all antibiotics were prepared by dissolving accurate quantities of the powdered standards in 1 ml ultra pure water and then diluted with acetonitrile. Mass concentration of standard solutions was 500 μg/ml. Stock solutions were stored protected from light at 4°C. Working standard solutions were made by diluting the stock standard solutions with acetonitrile so that their concentration was 5 μg/ml. Mixtures were made by mixing 1 ml of each working standard solution. EDTA was analytical reagent grade. Water was ultra pure.

Chromatographic plates 10x20cm, Kiselgel 60 F_{254} (Merck, Darmstadt, Germany), 20x20 Silica gel H, 25 μm, binder free, Anatech, HPTLC 20x10 silica gel 60 F_{254}, (Merck, Darmstadt, Germany), were used for method optimization. For solid-phase extraction 6cc/500 mg Oasis hydrophilic-lipophilic balance (HLB) cartridges (Waters, Milford, Massachusetts) were used.

Sample Preparation

Water samples that were used as a blank were taken upstream from the place for sampling. Both, blank water and water for analysis were collected in amber glass bottles.

Prior to extraction, water used in this study was filtered through black Whatman filter to eliminate the suspended matter. The samples were stored at 4°C until SPE extraction. Before extraction, total concentration of calcium and magnesium was determined, followed by addition of the appropriate amount of 0.01 M EDTA to prevent binding of the antibiotic to the calcium and magnesium. The spiked water samples were prepared by addition of 1 ml of stock standard solution of each antibiotic to 100 mL of water. Before the extraction water samples and spiked water samples were filtered through P/N 0.2 μm 47 mm GHP membrane filter. The HLB SPE cartridges previously used for tetracycline and fluoroquinolone determination reported in literature, were used (Ternes, Bonerz and Schmidt, 2001).

Solid-Phase Extraction

The antibiotics were extracted and pre-concentrated on 6cc/500 mg (Waters) HLB cartridges on the apparatus for solid-phase extraction. Before water application, the cartridges were conditioned with 5 mL of each methanol, and water pH 3.0.

The pH of water samples and water for preconditioning and washing steps was adjusted to 3.0 with hydrochloric acid. The samples were applied to the cartridge and the flow was kept at 3 mL min^{-1}.
After extraction cartridges were washed with 2 ml of ultra pure water to remove the residue of EDTA and dried under vacuum for 5 minutes to remove water excess. Elution of the antibiotic was performed with 3 ml of acetonitrile. The filtrates were evaporated under a stream of nitrogen to a volume of 1 ml. 10 µl of the filtrate was applied on the chromatographic plate.

Thin-Layer Chromatography
Analysis was performed according to the procedure described by Dong *et al* (1999) with the modification of the mobile phase. Prior to the analysis, three different chromatographic plates 10x20cm, Kiselgel 60 F_{254}, 20x20 Silica gel H, 25 µm, binder free, HPTLC 20x10 silica gel 60 F_{254} were impregnated 24 hours with 10% EDTA solution pH 9.0 and then were dried at room temperature for 2 hours, and in oven for 30 minutes at 105°C. On the plate prepared as described previously, aliquots of 10 µl of the each working solutions, mixture, blank and water samples were applied with micropipette in the form of spot.
The plates were developed in a closed glass Camag double-trough chamber containing mobile phase water/methanol/dichloromethane (6/35/59) (v/v) with previous saturation. The system was maintained until the mobile phase ascended to a point 7 cm above initial spots. After development, the plates were air dried and the chromatograms were visualized under UV light at 254 nm and 366 nm.

Surface Water Samples Analysis
The described method was applied to the determination of tetracycline, oxytetracycline, chlortetracycline, enrofloxacin and ciprofloxacin in surface water samples collected downstream from two fish farms. The volume of pre-filtered and acidified (pH 3) surface water samples was 250 ml. Before extraction, appropriate amount of 0.01 M EDTA was added to the water samples. The samples were applied to Oasis HLB cartridges. Antibiotics were eluted with 3 mL acetonitrile and filtrates were evaporated to 1 mL. Aliquot of 10 µL were applied on the TLC plate.

RESULTS AND DISSCUSION

Tetracyclines and fluoroquinolones have a very strong tendency to form complexes with trace metals in the adsorbents used, which cause lower separation performance because of spots tailing. Before use, the plates listed in section Materials and Methods, were impregnated with a 10% EDTA solution pH 9.0. Analysis was performed with several mobile phases that are described in the literature for the individual identification of tetracycline or fluoroquinolone (Dong *et al.* 1999, Wang, Chen, and Fan, 2001, Feng and Dung, 2004, Oka, Ito and Matsumoto 2000, British Pharmacopoeia, 2012).

The best simultaneous separation of all antibiotics was achieved on HPTLC plate 20x10 silica gel 60 F_{254} with a mobile phase water/methanol/dichloromethane (6/35/59 v/v/v) and these conditions are chosen for the analysis of surface water samples. (Figure 2).

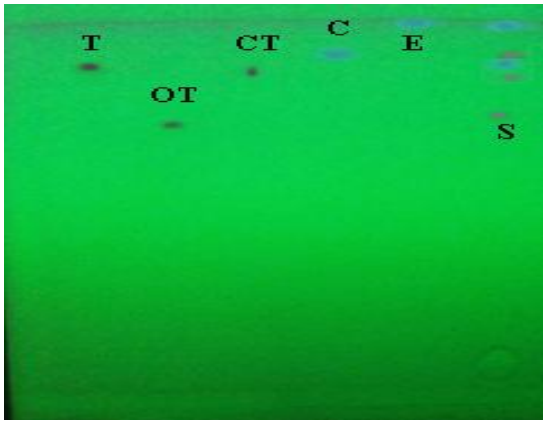


Figure 2: TLC chromatogram of antibiotic standards and mixture of standard substances (S)

Chromatographic plates were evaluated at λ=254 nm and λ=366 nm. Identification was done by comparison of Rf values of antibiotic standards and mix of standard substances (Table 1).

Table 1: Rf values of antibiotic standards and mixture of standard substances

Compds	Rf
tetracycline	0.57
oxytetracycline	0.35
chlortetracycline	0.45
ciprofloxacine	0.80
enrofloxaine	0.97
mix	0.37; 0.42; 0.56; 0.57; 0.97;

Among the five pharmaceuticals examined in this study, none were found in samples (S_1, S_2) used for analysis (Figure 3).

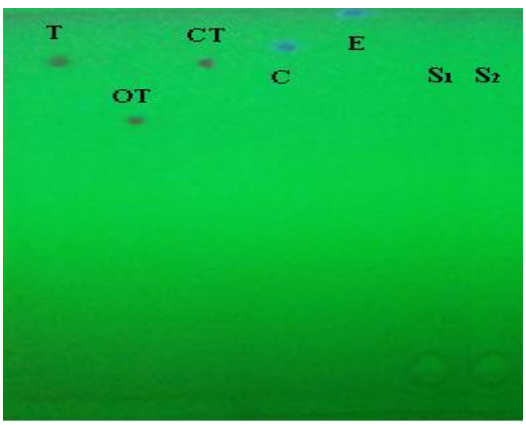


Figure 3: TLC chromatogram of antibiotic standards and water samples (S_1, S_2)

CONCLUSION

Solid-phase extraction followed by HPTLC-UV determination has been proposed for simultaneous identification (screening) of the tetracycline, oxytetracycline, chlortetracycline, enrofloxacin and ciprofloxacin. Identification were based on Rf values and UV detection. Limit of detection of proposed method was 50 ng per spot. Proposed method can be applied for screening of investigated antibiotics in water samples where antibiotic concentration is equal or higher than 5 μg/ml.

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Summary/Sažetak

Provedena je simultana identifikacija antibiotika tetraciklina, oksitetraciklina, hlortetraciklina, ciprofloksacina i enrofloksacina u površinskoj vodi. Postupak se zasnivao na ekstrakciji na čvrstim fazama (SPE), razdvajanju i identifikaciji primjenom hromatografije na tankom sloju (TLC). Razdvajanje je provedeno na HPTLC silikagel 60 F254 pločama, uz mobilnu fazu voda/metanol/dihlormetan (6/35/59) (v/v). Ploče su prethodno impregnirane sa 10%-tnom otopinom EDTA pH 9,0, sušene u horizontalnom položaju najmanje dva sata na sobnoj temperaturi, a zatim se u sušnici na 105°C 30 minuta, neposredno prije upotrebe. Antibiotici su ekstrahirani primjenom OASIS HLB 6cc/500 mg kertridža. Volumen od 10 µl uzoraka vode i standardnih otopina apliciran je na ploču. Nakon razdvajanja, ploče su osušene na zraku, a hromatogrami su vizualizirani pod UV lampom na λ=254 nm i λ=366 nm. Rezultati su pokazali da se predložena metoda može primijeniti za simultanu identifikaciju ispitivanih antibiotika u uzorcima vode, u kojima su njihove koncentracije jednake ili veće od 5 µg/ml.