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Screening method for the determination of selected tetracyclines in water by liquid chromatography with diode array detector

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Abstract

A chromatographic procedure for determination of oxytetracycline (OXT), tetracycline (TC), chlorotetracycline (CTC), and doxycycline (DC) in water samples was developed and was applied for the analysis of water samples collected from poultry and pig farms and environmental water samples. Samples were acidified with trifluoroacetetic acid to pH 3 and further purified by solid phase extraction using Oasis HLB cartridges. The samples were dried up and redissolved in the mixture of oxalic acid and methanol. Separation was performed on reserved phase column (Phenomenex column C_{18} , 250 mm × 4.6 mm, 5 µm) by multistep gradient elution, and detection was carried out at 360 nm for OTC and TC, 370 nm for CTC, and 350 nm for DC. The tetracyclines were eluted with the mobile phase of 0.05 M oxalic acid (pH 2.5), acetonitrile, and methanol. This method provided average recoveries of 83.53% to 108.59%, with coefficient of variations (CVs) of 2.41% to 8.64% in the range of 10 to 1000 µg/L OTC, TC, CTC, and DC in water. The linearity for the tetracyclines was determined by HPLC-DAD in the range 10 to 1000 µg/L, with the correlation coefficient (R) > 0.99. The LOD and LOQ for the tetracyclines in water samples ranged from 1.51 to 4.00 and 2.51 to 5.93 µg/L, respectively.

Key words: water, tetracyclines, HPLC-DAD, SPE.

Introduction

Tetracyclines (TCs) are an important group of antibiotics. Since the first member of the tetracycline family, chlorotetracycline (CTC), was isolated from *Streptomyces aureofaciens* in 1944 (26), several TCs, such as tetracycline (TC), oxytetracycline (OTC), doxycyline (DC), and chlorotetracycline have been applied for treatment of diseases of animals and humans. Tetracyclines are broad-spectrum antibiotics active against most Gram-positive and Gram-negative bacteria. They are actively transported into the cells of susceptible bacteria where they bind to the 30S ribosomal subparticle. In this way, protein synthesis is inhibited, which explains their bacteriostatic effect (14). OTC, TC, CTC, and DC are commonly used in food-producing animals, because of their broad-spectrum activity and low production costs (3).

Since 2006, when the European Commission introduced the ban for the use of antibiotic growth promoters, antibiotics can be administrated by injection, as an addition to feed (medicated feedingstuffs) or water on the request of a veterinarian. However, cases of dishonest breeders, who administrate antibiotics in sub-therapeutic doses in feed and drinking water to accelerate animal growth, still occur.

Majority of chemotherapeutics are designed to be persistent, so that they retain their chemical structure long enough to act as chemotherapeutics, and this coupled with their continual input, may enable them to remain in the environment for a significant period. Chemotherapeutics have been found in a wide range of environmental samples including surface, ground, and drinking water (24).

Administration of antimicrobial substances for a long period may contribute to acquisition of the resistance of microorganisms, allergic reactions or toxic effects, and the presence of residues of antibiotics in edible tissues of the animals, as well as their presence in the environment: soil and water. Therefore, controlling the use of antibiotics in the veterinary

medicine is an important matter to protect the health of animals and customers.

There are several analytical methods used to detect tetracyclines with different matrices, for example liquid-chromatography (LC) and fluorescence detection (13, 24), as well as capillary electrophoresis (CE) using laser-induced fluorescence detection (21). LC with UV detection (5, 7, 9, 16, 19) has been also employed. Nowadays, liquid-chromatography coupled with mass spectrometry (LC-MS) (9, 15) and tandem mass spectrometry (3, 9, 10) seems to be the technique of choice for analysis of this group of substances. However, mass instruments are still quite expensive and are not readily available for chemists in testing laboratories.

This paper describes a method for detection of OTC, TC, CTC, and DC in drinking and environmental water samples. The analytes are preconcentrated by SPE and analysed by HPLC with diode array detector (DAD). The coupling of SPE with LC-DAD results is a selective and sensitive analytical method for monitoring TCs in the water, which is proposed as a simple and more economic alternative for MS based methods.

Material and Methods

Reagents and chemicals. Oxytetracycline hydrochloride, tetracycline hydrochloride, doxycycline hyclate, and chlorotetracycline hydrochloride were purchased from Sigma-Aldrich (USA), and all standard solutions were prepared using HPLC-grade methanol from Merck (Germany). Oxalic acid dihydrate was from Chempur (Poland), trifluoroactetic acid (TFA) was obtained from Sigma Aldrich (USA). Sodium hydroxide was from (POCH) Poland. Analytical grade solvent acetonitrile was from Merck (Germany). SPE cartridges Oasis HLB (60 mg, 3 mL) from Waters (Milford, USA). All solutions, including electrolytes, were prepared using purified Milli-Q water generated by a Milli-Q Plus Water Purification System (Millipore, USA).

HPLC-DAD analysis. The instrumental analysis was performed using HP 1100 Series chromatograph (Agilent Technologies, USA) equipped with solvent degasser, auto-sampler with 100 µL loop, quaternary pump, column thermostat, and diode array detector. The chromatographic separation was accomplished with gradient elution on column 250 mm × 4.6 mm, 5 µm (Phenomenex, USA). The flow rate was 0.8 mL/min, and the column thermostat was set at 30ºC. The injection volume was 100 µL. The UV detection was monitored at 360 nm for OTC and TC, 350 nm for DC, and 370 nm for CTC. The mobile phase consisted of 0.05 M oxalic acid, pH 2.5 (A), acetonitrile (B), and methanol (C). The gradient elution was as follows: 0-5 min 77% A, 23% B, 5-20 min changed to 42% A, 34% B, and 24% C, and conditions returned to initial state and were held for 5 min.

Standard solution. The stock standard solution of OTC, TC, CTC, and DC (Fig. 1) was prepared by weighing 5 mg ± 0.1 mg of standard substances and dissolving it in 5 mL of methanol. The solution was stable for one month, stored at -20ºC.

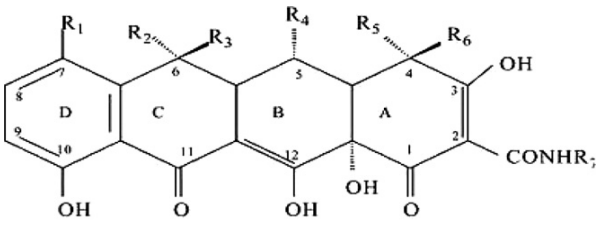

Fig. 1. Structure of tetracyclines

Calibration curves. Series of working standard solutions were prepared at the concentrations: 0.01, 0.05, 0.1, 0.5, and 1 µg/mL of OTC, TC, CTC, and DC in deionised water. These solutions were analysed by HPLC-DAD and calibration curves were plotted. The three linearity studies were compared and accuracy was calculated.

Samples. Samples of water were taken by the Veterinary Inspection from pig and poultry farms from different parts of Poland, and were delivered to the National Veterinary Research Institute in Pulawy for the official control. Water samples were stored at 2–6ºC.

Extraction procedure. Water samples were collected in glass bottles. Prior to extraction, drinking water samples were filtered with Whatman paper filters to eliminate suspended matter. Each water sample was acidified with trifluoroactetic acid to pH 3. Thirty millilitres of water sample was transferred to a 50 mL polypropylene centrifuge tube and centrifuged for 20 min at 4000 × g.

Clean–up. For the clean-up step the Solid-Phase Extraction (SPE) apparatus (J.T. Baker) and Oasis HLB cartridges (100 mg, 3 mL) were used. The cartridges were conditioned with 4 mL of methanol, followed by 4 mL of deionised water. Twenty five millilitres of water sample were loaded on the cartridge and next washed with 4 mL of water, which was then discarded. The chemotherapeutics were eluted with 3 mL of methanol. The elute was evaporated to dryness under nitrogen stream and the residue was reconstituted in 1 mL of 0.05M oxalic acid: methanol mixture (9:1 v/v).

Validation procedure. The validation of the method was performed according to the recommendations of the Commission Decision 2002/657/EC. Parameters such as linearity, specificity, precision, accuracy, limits of detection (LOD), quantification (LOQ), and decision, as well as detection capability were established. The method was validated

for four tetracyclines on three concentration levels (10, 100, and 1000 µg/L).

Calibration curves for the OTC, TC, CTC, and DC were constructed. Linearity was tested by preparing matrix calibration curve in the range of 10–1000 µg/L. Blank water sample was fortified with standard solution of OTC, TC, CTC, and DC at five concentration levels: 0.01, 0.05, 0.1, 0.5, and 1 µg/mL.

Selectivity was confirmed by the absence of interfering peaks in the retention time windows of OTC, TC, CTC, and DC, calculated as ±5% of the relative retention times for TCs. These time windows were also used in the analyses of six negative water samples to determinate limits of LOD and LOQ, considering $x+3*SD$ and $x + k*SD$ respectively, where x is the average concentration of the analyte in the sample, calculated from the equation of the calibration curve, whereas SD is the standard deviation of the calculated concentration, and $k - 6$ or 10 depending on the lowest concentration of analyte that can be determined with an acceptable level of precision.

For the evaluation of precision (repeatability, within-laboratory reproducibility), as well as recovery, blank water samples were spiked with OTC, TC, CTC, and DC working standard solution to levels corresponding to 10, 100, and 1000 µg/L respectively. The experiments were carried out on three consecutive days. The repeatability was determined by fortifying six blank samples at each of three concentration levels (10, 100, and 1000 µg/L) with OTC, TC, CTC, and DC compounds. The samples were analysed on the same day with the same instrument and the same operators, and the CVs was calculated. The within-laboratory reproducibility was determined by fortifying other two sets of blank samples at the same concentration levels of the analysed compounds as for the repeatability and analysed on two different days with the same instrument and different operators. All CVs were calculated.

The CCα and CCβ were determined by the matrix calibration curve procedure. CCα was calculated with the statistical certainty of 1–α (α = 0.05) and CCβ was calculated with the statistical certainty of 1–β (β = 0.05).

Results

The validation parameters were estimated on the basis of in-house validation concept in accordance with Commission Decision 2002/657/EC. Matrix calibration curves were used for quantification. The specificity of the method was checked by analysing blank water samples and the specificity was 100% for all analytes, as no peak was detected at the retention time corresponding to each analyte. The recoveries were in the range between 83.53% and 108.59%. Precision (repeatability, and within laboratory reproducibility) of the procedure, as well as decision limit (CCα) and detection capability (CCβ) were determined by the matrix calibration curve procedure. The CCα and CCβ were from 32.01 µg/L to 69.03 µg/L and from 36.50 µg/L to 112.93 µg/L respectively. All parameters are shown in Table 1.

The chromatograms of spiked, blank sample, and animal drinking water samples supplied by the Veterinary Inspection, in which doxycycline was found at 40 µg/L are shown in Figs 2, 3, and 4. The stability of individual stock standard solutions stored at -20ºC remained for at least 6 months.

Table 1. Validation of the method

Parameter		Results			
Analite name		OTC	TC	CTC	DC
CCα (µg/L)		32.01	69.03	67.26	33.69
CCβ(µg/L)		36.50	85.92	112.93	56.38
LOD(µg/L)		2.60	4.08	1.64	1.53
LOQ(µg/L)		5.42	5.91	2.73	2.56
Correlation coefficient, R		1.00	0.99	0.99	0.99
Linearity (µg/L) (working range)		10–1000	10–1000	10–1000	10–1000
Recovery (%) (µg/L)	10	95.07	93.24	103.96	90.6
	100	96.35	86.36	83.53	103.58
	1000	100.50	101.76	105.96	108.59
Repeatability (%) (µg/L)	10	8.64	7.99	3.86	1.89
	100	2.41	4.54	3.38	2.91
	1000	3.65	4.47	2.54	2.76
Reproducibility (%) (µg/L)	10	7.67	8.40	9.61	7.50
	100	4.11	5.14	7.45	4.13
	1000	4.72	3.42	2.34	6.70

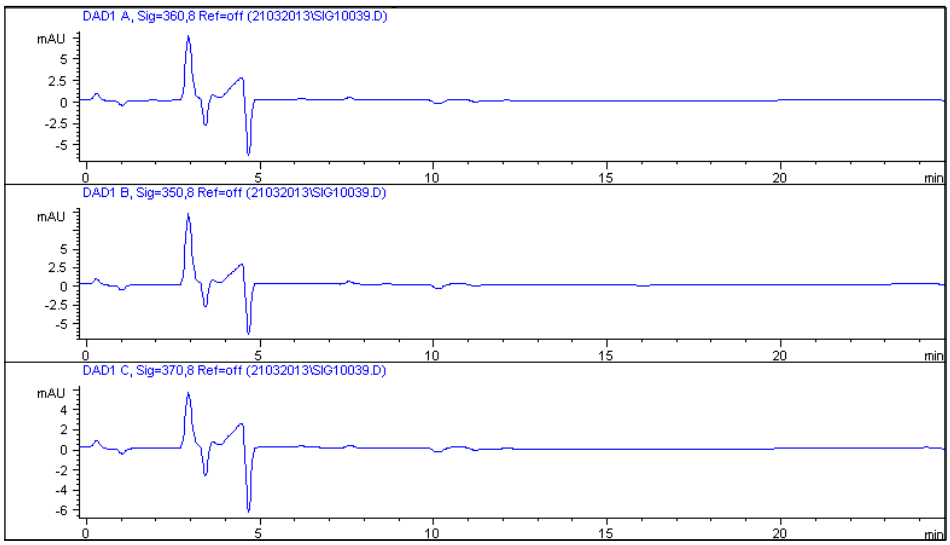

Fig. 2. HPLC chromatograms of blank water sample

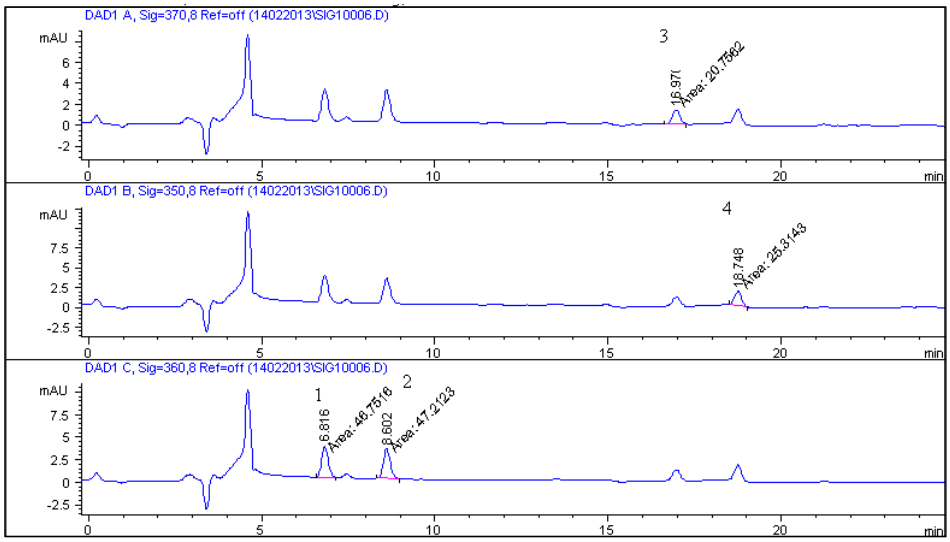

Fig. 3. HPLC chromatograms of water sample spiked with mixture of four TCs (10 µg/L) (b); 1 – oxytetracycline (λ=360 nm), 2 – tetracycline (λ=360 nm) , 3 – chlorotetracycline (λ=370 nm), 4 – doxycycline (λ=350 nm)

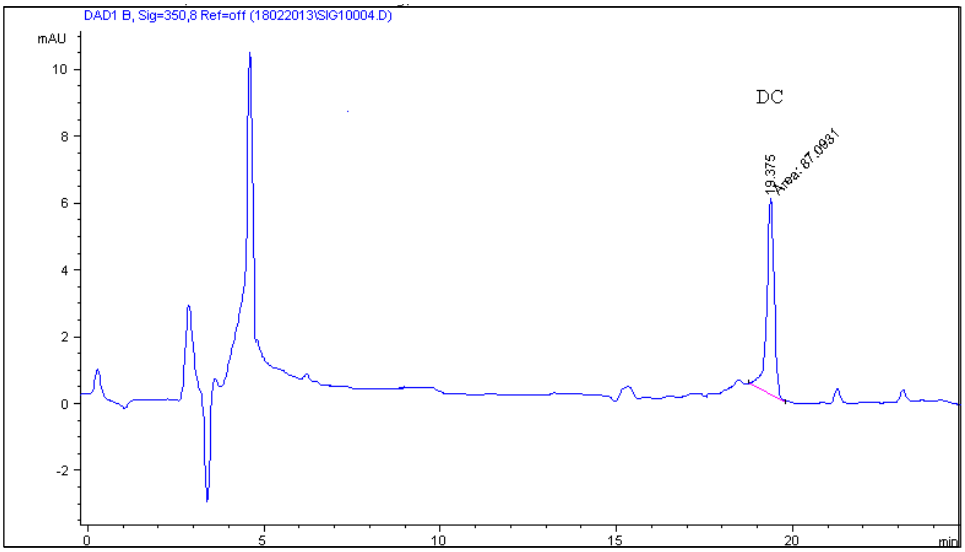

Fig. 4. HPLC chromatograms of water sample collected by the Veterinary Inspection, in which 40 µg/L of doxycycline (DC) was found

Discussion

A SPE followed by HPLC-DAD determination was proposed for simultaneous detection of the pharmaceutical compounds: oxytetracycline, tetracycline, doxycycline, and chlorotetracycline. The identification of the pharmaceuticals was based on the correlation of the retention times and UV spectra.

The critical step in the method development is usually sample preparation procedure, especially due to amphoteric properties of many pharmaceuticals. Since tetracyclines are amphoteric and unstable under strong acid conditions, pH of the sample solution is one of the most important control parameters. In methods of analysis of tetracyclines in the water samples, at the initial stage, water samples were acidified to pH 2.8 – 3.4 most commonly with hydrochloric acid (1, 11, 16, 22) or formic acid to receive a better extraction. On the basis of preliminary examinations, pH 3 of water samples was chosen as optimum. Therefore, all water samples were acidified with trifluoroacetic acid to pH 3.

Tetracyclines have been determined in a number of matrices such as milk and muscle (3, 8 19), fat (9), eggs (7), honey (25), plasma (19), pharmaceutical products (12), and water (15, 16). Blanchflower *et al.* (2) described the use of a glycine–HCl buffer for C18 extraction of tetracyclines from muscle and kidney samples. Walsh *et al.* (21) used a 0.1 M EDTA-McIlvaine buffer that resulted in more consistent recoveries for tetracyclines extracted from meat samples. Metal-chelating affinity chromatography (MCAC) has also been used for the clean-up of tetracyclines in food, serum, and urine samples (6). However, an additional desalting step of eluent from the MCAC column is required prior to analysis by LC-MS. Solid-phase microextraction (SPME) may be problematic for complex biological matrices due to its reduced loading capacity, and may not be convenient for routine analysis (26).

Solid-phase extraction (SPE) using the reversed-phase octadecyl (C18) cartridge has become routine in purifying and concentrating environmental contaminants (20). A great number of methods for extracting tetracyclines using C18 have been developed to replace more labour-intensive liquid-liquid extraction (18) and lyophilisation (11).

Yang Xiao *et al.* (30) presented a method for tetracyclines extraction from environmental water samples using a recently developed cartridge containing a macroporous poly-(divinylbenzene-co-N-vinylpyrrolidone) sorbent (Oasis HLB, Waters, USA). Major advantages of the cartridge include more rugged extraction, improved recovery for both polar and non-polar compounds in complex matrices, and greater capacity than reverse-phase silica based sorbent (4).

During the experiments, SPE extraction coupled with C18 and Oasis HLB columns was used to clean up and concentrate the extracts. The use of C18 columns provided lower recoveries of the analysed antibiotics and worse chromatographic images. Use of Oasis HLB cartridges resulted in more satisfactory results – reduced interferences of matrix and higher recoveries of tetracyclines. Nevertheless, literature often refers to reverse phase SPE, it was proved that amphoteric pharmaceuticals bind strongly to free silanol groups, thus they cannot be eluted with standard organic solvents, leading to worse recovery results. To handle such problem, functionalised SPE materials that separate analytes by their hydrophobic and polar properties, *e.g.* hydrophilic-lipophilic balance cartridges (HLB), appear to be more advantageous (1).

Many authors use C18 or C8 chromatography column for the separation of tetracyclines from water samples (16, 17, 26). The separation of analytes from water samples is usually performed using C18 and C8 chromatography columns, which is why, in this study these columns were tested. Better results of tetracyclines determination were obtained using the C18 column. This column enabled good separation and provided appropriate shapes of all peaks in the single run. Moreover, various solvents (oxalic acid, trifluoroacetic acid, acetonitrile, and methanol) and their volume proportion in mobile phase were tested to obtain the best resolution of the antibiotics. Acetonitrile, methanol, and 0.05 M oxalic acid, pH 2.5 were selected as the most efficient composition of mobile phase. The isocratic and gradient flow programmes were evaluated for the separation all of the tetracyclines, but isocratic system did not allow to separate the compounds, especially OTC and TC. Further improvement in the separation was obtained by application of mobile phase gradient. Selected experimental conditions enabled separation of four TCs within 25 min.

The rapid and satisfactory extraction of OTC, TC, CTC, and DC from water samples has been demonstrated. The chromatographic procedure was based on the use of conventional C18 column and elution with the conventional aqueous-organic phases. Adequate resolution of all TCs peaks was achieved within a relatively short time. Consequently, it can be stated that extraction and chromatographic procedures are useful for screening and quantification of the antibacterials in water.

The procedure was successfully validated according to commission Decision 2002/657/EC. The method is implemented for routine control of antibiotics in water. In the first half of 2013, the developed method was tested on 24 samples of drinking water for animals collected in the framework of official controls by the Veterinary Inspection, and in six samples the presence of doxycycline was demonstrated.

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