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Original article

Screening method for the determination of tetracyclines and fluoroquinolones in animal drinking water by liquid chromatography with diode array detector

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Abstract

A liquid chromatography – diode array detector (HPLC-DAD) procedure has been developed for the determination of oxytetracycline (OTC), tetracycline (TC), chlorotetracycline (CTC), doxycycline (DC), enrofloxacin (ENR), ciprofloxacin (CIP), sarafloxacin (SAR) and flumequine (FLU) residues in animal drinking water. This method was applied to animal drinking water. Solid-phase extraction (SPE) clean-up on an Oasis HLB cartridge allowed an extract suitable for liquid chromatographic analysis to be obtained. Chromatographic separation was carried out on a C18 analytical column, using gradient elution with 0.1% trifluoroacetic acid – acetonitrile – methanol at 30°C. The flow-rate was 0.7 mL/min and the eluate was analysed at 330 nm. The whole procedure was evaluated according to the requirements of the Commission Decision 2002/657/EC, determining specificity, decision limit (CCα), detection capacity (CCβ), limit of detection (LOD), limit of quantification (LOQ), precision and accuracy during validation of the method. The recoveries of TCs and FQs from spiked samples at the levels of 10, 100 and 1000 µg/L were higher than 82%. The developed method based on HPLC-DAD has been applied for the determination of four tetracyclines and four fluoroquinolones in animal drinking water samples.

Key words: tetracyclines, fluoroquinolones, water, solid-phase extraction, HPLC-DAD

Introduction

Fluoroquinolones (FQs) and tetracyclines (TCs) are two common classes of veterinary medicine products widely used in animal treatment. FQs are very important antibacterial agents as they are highly active against a wide range of Gram-negative and Gram-positive bacteria, giving a satisfactory effect in the treatment of severe intestinal and respiratory infections in poultry, pigs and calves (Brown 1996, Martinez et al. 2006). TCs are also broad spectrum antibiotics, active against most Gram-positive and Gram-negative bacteria and atypical bacteria such as chlamydiae, mycoplasmas, rickettsiae and protozoan parasites. These antibiotics are commonly used in animal treatment, because of their broad-spectrum

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activity and low production costs (Schnappinger and Hillen 1996).

However, the long-term administration of antimicrobial substances can contribute to the acquisition of resistance by microorganisms, allergic reactions or toxic effects and the presence of antibiotic residues in the edible tissues of the animals, as well as in the environment, i.e. soil and water. Therefore, the control of the use of antibiotics in veterinary medicine is an important matter in protecting the health of animals and consumers.

There are several analytical techniques used to determine TCs and FQs in different biological matrices, for example: gas chromatography (GC) (Moeder et al. 2000), capillary electrophoresis (CE) (Nozal et al. 1995, Hernandez et al. 2000, Hernandez et al. 2002) and high-performance liquid chromatography (HPLC) (Miao et al. 2002, Garcia Mayor et al. 2006, Denooz and Charlier 2008) with UV and fluorescence detection (Esponda et al. 2009, Patyra et al. 2014). Nowadays, liquid-chromatography coupled with mass spectrometry (LC-MS) and tandem mass spectrometry seem to be the techniques of choice for analysis of these groups of substances (Hirsch et al. 1998, Charlet et al. 2003, Gajda et al. 2013). However, mass detectors are still expensive and are not readily available for chemists in feed and water testing laboratories.

This study describes a method for the determination of four tetracyclines and four fluroquinolones in animal drinking water samples. The coupling of solid-phase extraction (SPE) with the HPLC-DAD resulted in a selective and sensitive analytical method for determination of TCs and FQs in animal drinking water, which is proposed as a simple and more economic alternative for MS based methods. The aims of the study were the development, optimisation, and qualitative and quantitative determination of the antibiotics in animal drinking water.

Materials and Methods

Reagents and chemicals

Oxytetracycline hydrochloride, tetracycline hydrochloride, doxycycline hyclate, chlorotetracycline hydrochloride, sarafloxacin, ciprofloxacin, enrofloxacin and flumequine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was obtained from Sigma Aldrich (St. Louis MO, USA). Sodium hydroxide was purchased from POCH (Poland). Analytical grade solvents, acetonitrile (ACN) and methanol (MeOH), were from Merck (Germany). All solutions, including electrolytes, were prepared using purified Milli-Q water generated by a Milli-Q Plus Water Purification System (Millipore, Bedford, MA, USA).

Instrumentation

The chromatographic system was the HP 1100 Series chromatograph (Agilent Technologies, USA) equipped with solvent degasser, auto-sampler with 100 µl loop, quaternary pump, column thermostat, fluorescence detector (FLD) and diode array detector (DAD) system. The chromatographic column was Agilent Eclipse XDB C18 (150 x 4.6 mm, 5 µm Agilent Technologies, USA).

Two SPE cartridges Oasis HLB (60 mg, 3 ml) of Waters (Milford, MA, USA) and BondElut C18 (500 mg, 3 ml) of Agilent Technologies (USA) were used. SPE manifold (J.T. Baker, USA) and a pump as a vacuum source were used.

Chromatographic conditions

A triple mobile phase with a gradient elution was used. Solvent A was Milli-Q water with 0.1% TFA, solvent B was ACN and solvent C was MeOH. The gradient was as follows: 0-4 min. 84% A, 16% B, 0% C; 4-15 min. changed to 69% A, 25% B, and 6% C; 15-20 min. 62% A, 30% B, 8% C; 27-30 min. 84% A, 16% B, 0% C and conditions returned to initial state and were held for 3 min. The flow rate was 0.7 mL/min, and the column thermostat was set at 30°C. The injection volume was 35 µl and all compounds were eluted within 33 min. The diode array detector was used to optimize the separation and it was set at 330 nm.

Standard solutions

The stock standard solutions were prepared by weighing 5 mg±0.1 mg of standard substances of OTC, TC, CTC, DC, SAR, and ENR and dissolving in 5 ml of methanol, FLU was dissolved in acetonitrile and CIP in a mixture of methanol and 1M sodium hydroxide (99:1 v/v). The solutions were stable for one month, stored at -18°C.

Sample preparation

Animal drinking water samples were collected in glass bottles and stored at 2-6°C until analysis. Prior to extraction they were acidified with trifluoroacetic

acid to pH 3. Fifty millilitres of water sample was transferred to a 50 mL polypropylene tube and centrifuged for 20 min at 4000×g.

Cleanup

For the clean-up step the SPE apparatus and Oasis HLB cartridges (100 mg, 3 mL) were used. Prior to sample loading, the hydrophilic-lipophilic balance cartridges were preconditioned successively with 4 mL of methanol and 4 mL of deionized water. Sample volumes of 10, 25 and 40 mL were tested for the extraction and finally 40 mL was chosen as the most efficient amount. After percolation, the cartridges were washed with 4 mL of deionized water and vacuum dried for 1 min. The antibiotics were eluted with 3 ml of methanol. The eluate was evaporated to dryness under nitrogen stream and the residue was reconstituted with 0.5 mL of 0.1% trifluoroacetic 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, limit of detection and quantification, decision limit and detection capability were established. The method was validated for four tetracyclines and four fluoroqiunolones at three concentration levels: 10, 100 and 1000 µg/L.

Calibration curves for the OTC, TC, CTC, DC, SAR, CIP, ENR and FLU were constructed. Linearity was tested by preparing matrix calibration curves in the range of 10 – 1000 µg/L.

Selectivity was confirmed by the absence of interfering peaks in the retention time windows of OTC, TC, CTC, DC, SAR, CIP, ENR and FLU calculated as ±5% of the relative retention times for TCs and FQs. These time windows were also used in the analyses of six negative water samples to determinate limits of detection (LOD) and quantification (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, DC, SAR, CIP, ENR and FLU standard solutions to levels corresponding to 10, 100 and 1000 µg/L. The experiments were carried out on three consecutive days. The repeatability was determined by fortifying six blank samples at each of the three concentration levels. The samples were analyzed on the same day with the same instrument and the same operator, and the coefficient of variations (CV) was calculated. The within-laboratory reproducibility was determined by fortifying two other sets of blank samples at the same concentration levels of the analyzed compounds as for the repeatability and analysed on two different days with the same instrument and different operators. The overall CVs were calculated.

The decision limit (CCα) and detection capability (CCβ) were determined using 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).

Sample collection

Samples of animal drinking water were taken by the Veterinary Inspection from pig and poultry farms of different parts of Poland and delivered to the National Veterinary Research Institute in Pulawy, in the framework of the official control. Water samples were delivered in plastic bottles and dark plastic envelopes protecting against UV rays and then stored at 2 – 6°C.

Results

The validation parameters were estimated on the basis of the 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 other peaks were detected at the retention time corresponding to each analyte.

The accuracy of the method and repeatability were evaluated by analyzing OTC, TC, CTC, DC, CIP, ENR, SAR and FLU in spiked water at levels of 10, 100 and 1000 µg/L (six replicates for each level). The results (Table 1) show that the assay recovery was in the range of 82.1 to 114.7% and CVs were less than 14.5%. The recovery precision of the method was calculated using 10, 100 and 1000 µg/L spiked water samples. The results showed that the CVs of OTC, TC, CTC, DC, CIP, ENR, SAR and FLU peak areas were less than 18.5% (Table 1). The reproducibility was established by analyzing other sets of samples spiked at the same concentration levels as for the repeatability.

Table 1. Recoveries, recovery precision and CVs of TCs and FQs in water.

Analyte	Fortified concentration (µg/L)	Rates of recovery (%)			
		Water samples			
		Recovery (%)	Repeatability CV (%)	Reproducibility CV (%)	Recovery precision (%)
Oxytetracycline	10	112.6	2.9	6.9	
	100	86.7	9.7	10.3	13.7
	1000	93.8	5.6	7.6	
Tetracycline	10	101.3	6.9	10.8	
	100	94.7	3.7	9.5	3.4
	1000	99.1	9.2	8.9	
Chlorotetracycline	10	105.3	8.7	8.9	
	100	89.6	6.8	7.2	8.9
	1000	91.8	7.8	7.5	
Doxycycline	10	114.7	10.3	14.4	
	100	82.1	6.6	10.0	18.5
	1000	87.1	8.0	8.9	
Enrofloxacin	10	106.1	8.4	8.0	
	100	82.6	13.5	14.4	12.5
	1000	98.9	4.1	5.6	
Ciprofloxacin	10	107.4	11.3	10.1	
	100	97.2	5.6	12.4	4.9
	1000	103.4	3.7	4.7	
Sarafloxacin	10	101.6	5.9	7.8	
	100	97.2	10.9	10.6	11.5
	1000	106.6	3.0	5.9	
Flumequine	10	105.1	12.1	14.5	
	100	92.86	7.7	12.5	6.7
	1000	94.82	5.1	6.9	

Table 2. Limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ) values of TCs and FQs in water samples.

Analytes	LOD, (µg/L)	LOQ, (µg/L)	CCα, (µg/L)	CCβ, (µg/L)
OTC	3.5	4.4	16.1	39.0
TC	4.3	6.3	26.0	63.0
CTC	6.3	8.2	22.0	53.3
DC	6.5	7.9	22.7	54.9
ENR	6.2	7.8	12.6	30.6
CIP	5.6	7.5	10.7	25.9
SAR	6.3	7.3	9.8	23.7
FLU	5.1	6.6	14.6	35.4

LOD and LOQ values for the TCs and FQs in water ranged from 3.5 to 6.5 and from 4.4 to 8.2 µg/L, respectively. The CCα, and detection capability CCβ were from 9.8 to 26.0 µg/L and from 23.7 to 63.0 µg/L, respectively. The parameters are presented in Table 2. This method has been shown to be appropriate for all the analytes with acceptable accuracy and precision. Representative chromatograms of the samples spiked with the mixture of four TCs and FQs and the blank samples are shown in Fig. 1.

Discussion

A SPE followed by HPLC-DAD detection was proposed for simultaneous determination of the antibacterial substances: OTC, TC, CTC, DC, ENR, CIP, SAR and FLU. The identification of the pharmaceuticals was based on the correlation of the retention times and UV spectra.

The critical step in method development is usually sample preparation procedure, especially due to the

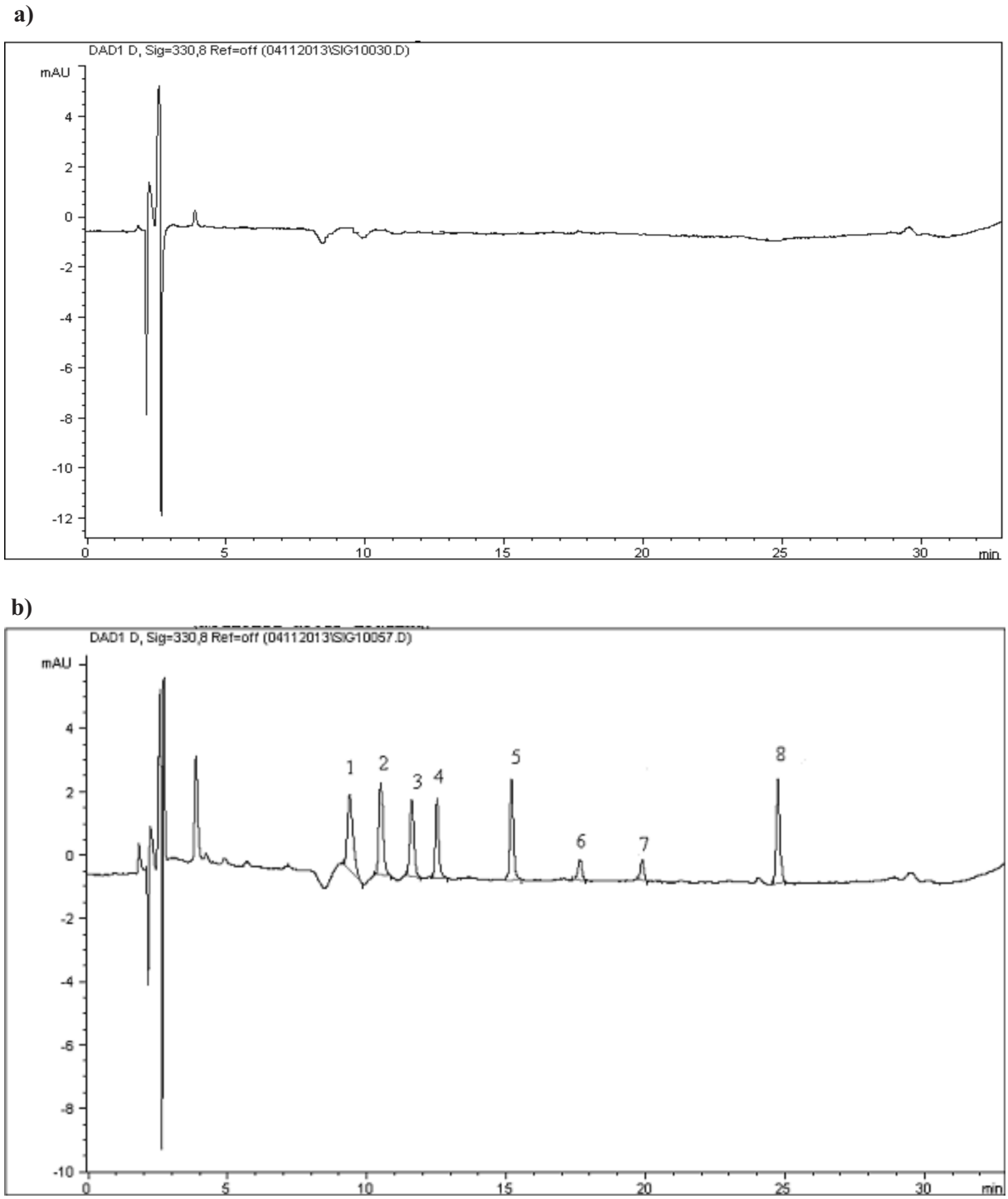

Fig. 1. Representative chromatograms of (a) blank water sample and (b) water sample spiked with mixture of oxytetracycline (1), ciprofloxacin (2), tetracycline (3), enrofloxacin (4), sarafloxacin (5), chlortetracycline (6), doxycycline (7), flumequine (8), at a concentration of 10 μg/L.

amphoteric properties of many pharmaceuticals. TCs and FQs have different pKa values, which should be considered during the pH adjustment of the sample. As the initial stage in various methods of analysis of tetracyclines in water samples, the water samples were acidified to pH 2.8 – 3.4 most commonly with hydrochloric or formic acid to obtain better extraction (Babić et al. 2006, Jia et al. 2009). On the other hand, recoveries of FQs were higher when the basic medium (pH = 7) was used (Esponda et al. 2009). Acidifying the sample to pH 3 with trifluoroacetic acid was chosen due to increased recoveries of TCs and acceptable recoveries of FQs. As pharmaceuticals appear at low concentrations in environmental water, enrich-

Table 3. Comparison of recoveries (%) obtained with three replicates of different sample volumes on C18 and Oasis HLB cartridges.

Volume	C18								Oasis HLB							
	OTC	TC	CTC	DC	ENR	CIP	SAR	FLU	OTC	TC	CTC	DC	ENR	CIP	SAR	FLU
10 mL	57	71	80	75	88	82	80	85	89	88	76	71	89	91	80	92
25 mL	78.2	89	83	79	91	87	82	87	94	95	87	89	99	98.4	98.7	99.1
40 mL	88	91	87	75	92.2	90.9	93.1	100	93	96	91	88	98	99	102	100

ment steps are needed. Therefore, solid-phase extraction (Kamel et al. 1999, Lindsey et al. 2001, Terens et al. 2001, Grandos et al. 2005, Babić et al. 2006) has been the preferred technique, although liquid-liquid extraction and solid-phase microextraction (Moeder et al. 2000) were used in some cases.

The SPE conditions were tested and optimized by analyzing two different SPE cartridges: BondElut C18 and Oasis HLB, and three replicates of different volumes (10, 25 and 40 mL) of tap water samples spiked at levels 10, 100 and 1000 µg/L. The use of C18 cartridges for the purification and concentration of the TCs reduced recoveries, probably due to association of tetracyclines with the silanol groups. Improvement of TCs recoveries can be obtained by adding a chelating agent such as EDTA or oxalic acid. Polymeric sorbent (Oasis HLB) can be used to retain hydrophilic and hydrophobic compounds with great capacity, moreover it does not contain silanol groups that can interact with TCs, as in the case of columns packed with C18. Additionally, the use of the polymer column for the extraction of FQs and TCs is recommended by a number of authors (Zhu et al. 2001, Grandos et al. 2005, Hao et al. 2006, Jia et al. 2009). The recoveries obtained for different samples volumes and cartridges are shown in Table 3. The best recoveries were obtained using Oasis HLB cartridge and 40 mL sample volume.

The literature (Kummerer 2001, Lindsay et al. 2001, Ramos et al. 2003, Seifertova et al. 2008) describes many methods for the determination of fluoroquinolones in different matrices with the use of a fluorescence detector due to the natural fluorescence properties of these compounds; however, tetracyclines must be first derivatizated, and this increases the time of sample preparation and time of analysis. Therefore, the diode array detector is most commonly used for the simultaneous analysis of these two groups of compounds. In the method developed here, in order to facilitate analysis (especially data processing), a single wavelength, which would allow the detection of all analyzed compounds was sought, and a wavelength of 330 nm was the best match for all analyzed TCs and FQs. The use of this wavelength improved the resolution of the analytes and no interferences from matrix compounds were observed.

Many authors separated TCs and FQs with the use of C18 columns (Esponda et al. 2009, Lilenberg et al. 2009, Patyra 2014), even though TCs form chelates with divalent and trivalent cations, and they additionally strongly interact with the silanol groups, binding to the chromatographic stationary support. Free silanol groups and metal impurities in column materials cause peak broadening and tailing. Use of oxalic acid as a component of the mobile phase of pH 2 or 3 decrease the binding of tetracyclines to the column stationary phase but causes clogging of the column and drifts of the retention times of the analyzed substances. For this reason, in the developed analytical method, separation of FQs and TCs was carried out using 0.1% trifluoroacetic acid, acetonitrile and methanol, with gradient elution. The time of analysis was 33 minutes. Applied mobile phase allowed good separation of the eight compounds.

The rapid and satisfactory extraction of OTC, TC, CTC, DC, SAR, ENR, CIP and FLU from water samples has been demonstrated. The chromatographic procedure was based on the use of a conventional C18 column and elution with conventional aqueous-organic mobile phases. This method was implemented for the routine control of antibiotics in animal drinking water. In 2013, 24 samples of drinking water for animals collected during official inspection by the Veterinary Inspection were tested with the use of the developed method. The presence of doxycycline in six samples and enrofloxacin in nine samples was detected. The existence of low concentrations of antibiotics in most cases is the result of contamination of water supply networks after prior use of drugs in therapeutic doses.

The developed procedure is simple, rapid, inexpensive, and the obtained validation results indicate that it can be used for detection of low concentrations of FQs and TCs for inspection purposes. Adequate resolution of all FQs and 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.

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