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Bull Vet Inst Pulawy 56, 329-333, 2012
DOI: 10.2478/v10213-012-0058-5

DETERMINATION OF CHLOROTETRACYCLINE AND DOXYCYCLINE IN MEDICATED FEEDINGSTUFFS BY LIQUID CHROMATOGRAPHY

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Received: May 16, 2012 *Accepted: September 7, 2012*

Abstract

High performance liquid chromatography method with diode array detection (HPLC-DAD) was developed and optimised for the determination of tetracyclines (TCs) in medicated feedingstuffs. The extraction of the analyte from feedingstuffs was performed with Na_2EDTA-McIlvaine buffer (pH 2.5 and pH 4). The extracts were cleaned up by solid phase extraction using octadecyl cartridges (C18). The samples were dried up and redissolved in the mixture of oxalic acid and methanol. Separation was performed on reserved phase column (Phenomenex C18, 250 x 4.6 mm, 5 µm) by multistep gradient elution, which provided better chromatographic separation. The analysis was performed at a wavelength of 390 nm. Validation study of the method revealed that all obtained calibration curves showed good linearity R= 0.9985 for doxycycline (DC) and R= 0.9981 for chlorotetracycline (CTC) over the range of 25–2,000 mg/kg. The analytical procedure was successfully adapted for quantitative determination of DC and CTC in medicated feedingstuff samples. Validation included determination of specificity, linearity, and repeatability. Mean recovery for spiked samples was 93.1% for CTC and 93.2% for DC. The results of validation of the analytical procedure proved that presented method is efficient, precise and useful for quantification of DC and CTC in medicated feedingstuffs.

Key words: medicated feedingstuffs, doxycycline, chlorotetracycline, liquid chromatography.

Tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacylo-tRNA to ribosomal acceptor (A) site (4). Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of Gram-positive and Gram-negative bacteria, atypical bacteria such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites (1, 9, 11).

In the production of medicated feedingstuffs, chlortetracycline (CTC) and doxycycline (DC) are usually used. Medicated feeds containing CTC and DC are mainly used in prevention and treatment of bacterial diseases of pigs and poultry. The antibiotics are used in the treatment of chronic respiratory disease, primary and secondary infections of the respiratory, digestive, and urinary tracts and multisystem infections in pigs, chickens, turkeys, and ducks. Since 2006, when the European Commission banned the use of antibiotic growth promoters, the demand for production of medicated feedingstuff has been observed. It became necessary to develop sensitive and rapid methods for the determination of active substances like DC and CTC in medicated feedingstuffs (5, 13).

One of the most widely used techniques for detection of most different classes of antibiotics in feedingstuffs are the microbiological tests. However, due to the risk of false positive results, they are not recommended for analysis of samples with complex matrix (2, 12). Furthermore, results of microbiological tests usually require confirmation by other methods allowing selective, accurate, quick detection and precise quantification of antibiotics in feedingstuffs. Specific analytical problems can be solved by means of instrumental chromatographic methods. Moreover, the level of quantification of such methods is much better than other techniques (12).

The need of the official control of homogeneity and appropriate therapeutic content of antimicrobial substances led to the development of instrumental methods of quantification. This paper presents simple and selective HPLC-DAD method for the determination of DC and CTC in medicated feedingstuffs. The aim of the study was the development, optimisation, and verification of the suitability of the method for qualitative and quantitative determination of the antibiotics in medicated feedingstuffs.

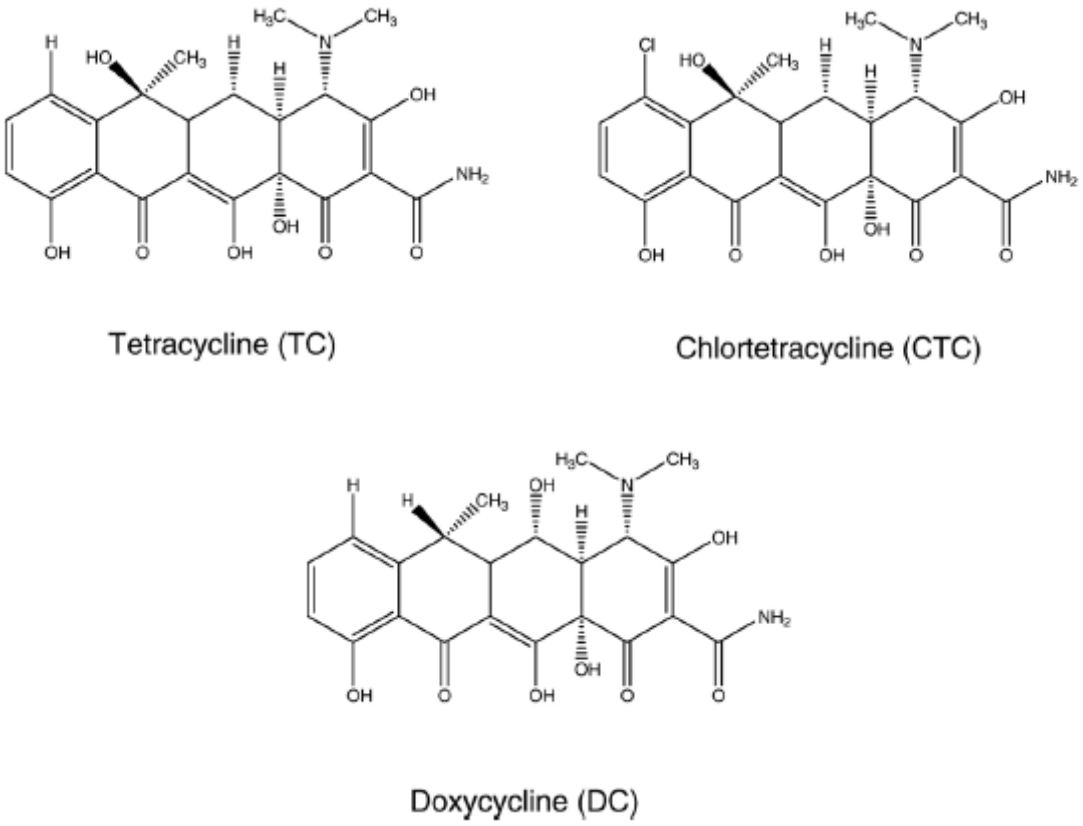


Fig. 1. Structures of tetracycline, chlorotetracycline, and doxycycline.

Material and Methods

Reagents and chemicals. Chlorotetracycline hydrochloride (CTC), doxycycline hydrochloride (DC), and tetracycline hydrochloride (TC) standards, methanol, acetonitryle, and ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA) were obtained from Sigma-Aldrich (USA). Citric acid was from Acros Organics (Belgium), *n*-hexane and disodium hydrogenphosphate dihydrate were obtained from POCh (Poland). Oxalic acid dihydrate (ACS) was from the Chempur Company (Poland). SPE cartridges C18 (500 mg, 3 ml) and nylon filters 0.45 µm were from Agilent Technology (USA). All chemicals were HPLC or analytical grade. Water was purified using Milli-Q system.

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 Luna column 250 x 4.6 mm, 5 µm (Phenomenex, USA). The flow rate was 1 mL/min, and the column thermostat was set at 30ºC. The injection volume was 20 µl. The UV detection was monitored at 390 nm. The mobile phase consisted of methanol (A), acetonitrile (B), and 5 mM oxalic acid (C). The gradient elution was as follows: 0-5 min held at 13% A, 12% B, 75% C, 5-20 min changed to 35% A, 20% B, and 45% C, and conditions returned to initial state and held for 5 min.

Standard solution. The stock standard solution of CTC, DC, and TC (IS) (10 mg/mL) was prepared by weighing 50.0 ±0.1 mg of standard substances and dissolving it in 10 ml of methanol. The solution was stable for one month, stored at 2-8ºC in amber glass. The stock standard solutions added to the samples of feed in appropriate amounts were used to plot a calibration curve and to study linearity.

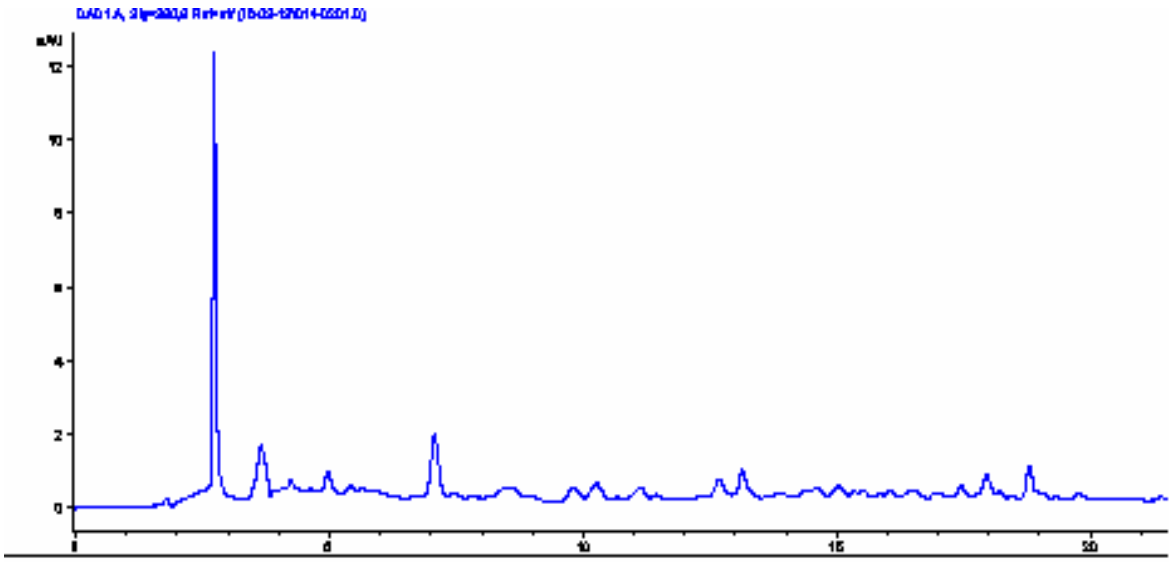
Extraction procedure. The feedingstuff sample was grinded. The internal standard (TC) was added to each sample to obtain concentration of 100 mg/kg, which corresponded to 13.2 µg/mL. Each sample was mixed with 15 ml of McIlvaine buffer-EDTA solution (pH 2.5) in a 250 ml Erlenmayer flask. The mixture was extracted for 20 min on a horizontal shaker, transferred to a 50 ml polypropylene centrifuge tube and centrifuged for 15 min at 3,500 x g. The supernatant was transferred to a new tube. Fifteen millilitres of McIlvaine buffer-EDTA mixture (pH 4) was added to the tube with remaining feed and the feed plug was resuspended using Vortex mixer. It was again centrifuged for 15 min at 3,500 x g and the supernatant was collected to the same tube as the first one, and 5 ml *n*-hexane was added. The mixture was shaken and centrifuged for 10 min at 2,500 x g.

Clean-up. For the clean-up step the Solid Phase Extraction (SPE) apparatus (J.T Baker) and C18 SPE cartridges (500 mg, 3 ml) were used. The cartridges were conditioned with 3 ml of methanol, followed by 3 ml of deionised water. After loading the sample, the cartridges were washed with 3 ml of 15% water solution of methanol, which was discarded. The drugs were eluted with 3 ml of methanol. The eluate was evaporated to dryness under nitrogen stream and the residue was reconstituted in 1 ml of mobile phase for HPLC analysis.

Validation. The analytical procedure was checked by evaluation of specificity, linearity, repeatability, and reproducibility. To determine the specificity of the method, blank samples of feedingstuff were analysed. Limit of detection (LOD) and limit of quantification (LOQ) were calculated on the basis of signal to noise ratio and were: S/N=3 for LOD and S/N=10 for LOQ. Four grams of drug-free feedingstuff sample (blank sample) was spiked with drug solution to obtain desired concentrations. To plot a calibration curve and to determine the linearity blank feedingstuff samples spiked at six concentration levels were used. The concentration levels: 25, 100, 250, 500, 1,000, and 2,000 mg/kg corresponded to concentration of 3.3, 13.2, 33, 66, 132, and 264 µg/mL of DC and CTC. The appropriate amount of internal standard (TC) at concentration of 100 mg/kg, which corresponded to 13.2 µg/mL, was added to each sample.

The repeatability, reproducibility, and recovery were determined by spiking blank feedingfstuff samples at three different concentrations: 25, 500, and 2,000 mg/kg, which corresponded to 3.3, 66, and 264 µg/mL. Each sample contained TC as the internal standard at the concentration of 100 mg/kg.

(a)



(b)

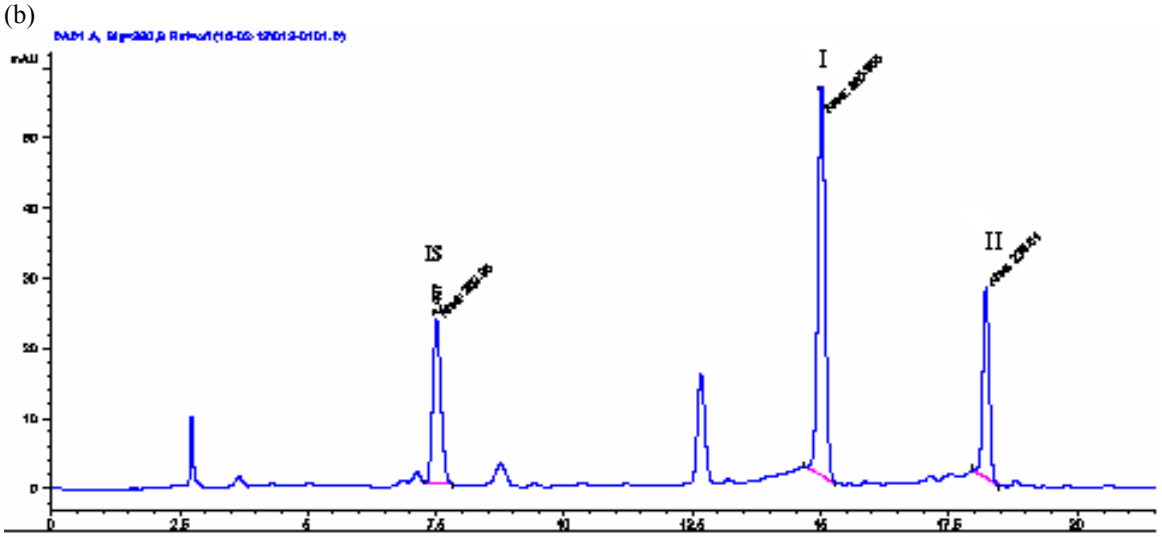


Fig. 2. Chromatograms of (a) blank feedingstuff sample, (b) feedingstuff sample spiked with chlorotetracycline (I) and doxycycline (II) at 250 mg/kg with internal standard (IS) tetracycline at 100 mg/kg.

Table 1
Results of validation of analytical procedure for the determination of CTC and DC in medicated feedingstuffs

Validation parameters	Results					
	DC			CTC		
Linear regression equation (y=ax+b)	Y=0.0747–0.2382			Y=0.0291+0.0238		
Correlation coefficient	0.9985			0.9981		
Working range (mg/kg)	25–2,000			25–2,000		
Limit of detection (mg/kg)	9.9			9.8		
Limit of quantification (mg/kg)	12.5			14.5		
Level of spiked samples (mg/kg)	25	500	2,000	25	500	2,000
Recovery (%)	85.1	96.2	98.4	91.4	92.5	95.5
Repeatability, CV%	7.32	0.18	2.47	2.74	1.82	0.92
Reproducibility, CV%	6.81	2.31	2.87	3.1	1.65	1.29

Results

Results of method validation are shown in Table 1. LOD and LOQ for CTC was determined as 9.8 and 14.5 mg/kg, whereas for DC was 9.9 and 12.5 mg/kg, respectively. The calibration curves were linear in the range: 25–2,000 mg/kg and the correlation coefficient was 0.9981 for CTC and 0.9985 for DC. Peaks of the analytes were sharp and symmetrical. Retention time for CTC was 14.832 min and for DC was 18.651 min. Fig. 2 shows the chromatograms of blank feedingstuff sample, and feedingstuff sample spiked with CTC and DC at concentration of 250 mg/kg. The recovery range was 91.4%-95.5% for CTC and 85.1% - 98.4% for DC.

Discussion

Currently, for the detection and quantitative determination of DC and CTC in medicated feedingstuffs, microbiological methods are mostly in use. However, there are some problems associated with incubation periods and with the lack of specificity of these methods. Therefore, it was necessary to develop a chemical method for quantitative determination of DC and CTC in medicated feedingstuffs.

Some analytical procedures for the determination of DC and CTC using chromatography techniques can be found in the scientific literature, nevertheless, they mostly consider biological matrixes, such as animal tissues, eggs, or milk. Only restricted number of articles is connected with feedingstuffs. That is why the attempt to develop a suitable analytical procedure for determination of DC and CTC in feedingstuff matrix was undertaken.

In spite of high concentration of DC and CTC in medicated feedingstuffs, the complexity of the matrix creates some difficulties with obtaining sufficient recovery of the active substances. In our preliminary study, we tried to find the most efficient way for sample extraction. Few extraction solvents were tested. On the basis of previously published articles, it was decided to test mixtures like: hydrochloric acid-acetone-water (1:8:6), acetonitryle-water (1:1), succinate buffer, 1 M hydrochloric acid, ethyl acetate, and McIlvaine buffer (pH 2, 3, and 4) (3, 6-8, 10, 14).

Extraction with organic solvents gave numerous impurity peaks. Evaporation of organic extracts decreased the level of impurities; however, this step is difficult to perform due to the presence of lipids. That is why it was decided to use the McIlvaine buffer of pH 2.5, 3, and 4, which gave less contaminated extract and relatively good recovery. Moreover, the use of buffer solution eliminated evaporation step before solid phase extraction (SPE), and improved deffating, which is necessary due to clogging problems of SPE cartridges. The best cleaning process found in the literature was achieved on C18 cartridge (7). In that study, 15% methanol in water was used for the wash step to remove impurities, which eluted near the retention time of DC and CTC, though, it was found that the use of 10% methanol for the washing step improved the recovery of active substances.

Most of the published methods considered chromatographic separation on C18 column, using isocratic elution of mobile phase. Acetonitryle or methanol, 1-buthanol, oxalic acid (pH 2) or sodium dodecylo sulphate or THF were mostly used as the mobile phase (3, 6-8, 14). In this study, gradient method was optimised on C18 column with the use 0.05 M oxalic acid. Use of gradient elution, gave much better separation of the analysed compounds and made the identification easier. The developed method gave good results for samples prepared in-house (spiked with the appropriate levels of DC and CTC) and samples delivered by manufacturers.

In conclusion, an analytical procedure based on ~~a~~ HPLC-DAD was presented. The procedure allowed the separation and quantification of doxycycline and chlorotetracycline in medicated feedingstuffs. The presented HPLC-DAD method is efficient, precise, and

useful for routine analysis. Results presented above prove the suitability of the method for the use in medicated feedingstuffs quality and homogeneity testing, and quantitative determination of active substances in the feedingstuffs.

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