RESEARCH ARTICLE **(Open Access)**

Determination of oxytetracycline, tetracycline and chlortetracycline in beef meat by HPLC-DAD detector in Albania

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Abstract

A study was conducted from June 2012 to May 2013 to estimate the proportion of tetracycline residue levels in beef at main slaughterhouses in Tirana, capital of Albania. A total of 37 beef muscle samples were randomly collected from slaughtered beef in the slaughterhouses. The samples were analyzed by using high performance liquid chromatography (HPLC), with Photo Diode Array detector (DAD). The detection limit of the method was calculated to be 25 µg/kg and the limit of quantitation was found to be 50 µg/kg. The recoveries of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) from spiked samples at three fortification levels were higher than 78% for all drugs. From 37 beef meat samples collected from different slaughterhouses of Tirana, only 4 samples showed detectable concentration of OTC residues but were lower than the maximum residue limits (MRLs) according to Commission Regulation (EU) No 37/2010. TC and CTC were absent in all samples. These levels were not able to induce risks to human health. However other studies are necessary to evaluate other drug residues in beef samples and to evaluate the hazards of these residues in relation with daily intakes and other related factors.

Keywords - Oxytetracycline, Tetracycline, Chlortetracycline, HPLC, residues

Introduction

Antibiotics are used in food producing animals not only to treat disease but also to maintain health and promote growth. Tetracyclines (TCs) are most widely used antibiotics in veterinary medicine in Albania due to its broad spectrum of antimicrobial activity, availability and low cost. Unauthorized use of these antibiotics, the failure to follow label directions or inappropriate withdrawal period of time before slaughtering of animals could lead to residues in food of animal origin, with potential adverse effects on human health. The overuse of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) in animal production or their residues in food system poses a potential allergic reaction in sensitized individuals, but sub-therapeutic and therapeutic levels may perturb human gut microflora by introducing resistant strains and altering the metabolic activity of the microflora, its resistant microorganisms barrier effects, and its ecological balance without any identified deleterious effects [8, 10, 11, 12, 13].

According to Commission Regulation (EU) No 37/2010, maximum residue limits (MRLs) set for OTC/TC/CTC (alone or combination) are 100 µg/kg in muscle tissues [6].

Several chromatographic methods have been employed successfully for the monitoring of TCs in tissue samples with different detection modes, such as UV-spectrophotometry, fluorescence, and mass spectrometry in the past [3, 4, 9]. All these procedures used a simple clean-up step by solid phase extraction (SPE) or matrix dispersion. The use of UV detector in residue analysis has low sensitivity, while mass spectrometry still has cost affair [2]. In general, PDA detection is sensitive and has wide scanning range. The objective of present study was to estimate the residue levels of tetracycline (oxytetracycline, tetracycline and chlortetracycline) in slaughtered beef from three different slaughterhouses in Central Albania by HPLC with PDA detector.

Material and methods

Samples were analyzed by high performance liquid chromatography following the techniques recommended by Agence Franaise de Securite sanitarie des aliments [1] for determination of

tetracycline residues in kidney and muscle by high performance liquid chromatography.

A- Homogenization: The samples were cut to fine pieces and 5 g of each sample was homogenized and diluted with 25 mL Mcllvaine buffer (mixed citrate/phosphate, pH 4.1 with EDTA). The mixtures were put in a high intensity sonicator for 10 min followed by shaking for 10 min.

B- Filtration: The homogenized samples were centrifuged 10 min at 10000 g. The supernatant was transferred to a beaker on a magnetic stirrer and 2.5 ml trichloroacetic acid was added slowly with constant stirring. It was centrifuged again for 5 minutes at 3000g. Then single GF/B filter paper was fixed in buchner funnel moisturized with McIlvaine buffer-EDTA and the supernatant was filtered through 0.45 µ filter.

C- Solid Phase Extraction: Oasis HLB cartridge was used as follows:

I. The cartridge was conditioned by 3 mL of methanol and then was rinsed by 2 mL of deionized water.

II. The prepared mixtures in stage B were loaded in a Oasis HLB cartridge.

III. The cartridge was washed with 2 mL of 5% methanol solution in water.

IV. TCs were eluted by 3 mL of HPLC grade methanol from the cartridge.

D. Evaporation and concentration: Eluted solutions (3 mL) were evaporated to be concentrated by a rotary evaporator. Concentrations and dilution factors were calculated and considered in the calculation of the real amount of drug residues in the sample after analysis. Chemicals, analytical standards of TCs, and Oasis HLB cartridge (WAT106202) were purchased from Merck (Germany), Sigma Chemical Co., and Waters (USA), respectively. HPLC performed using a Varian ProStar with 335 Photodiode Array Detector and Luna C8 (RP- C8) column 250 mm x 4.6 mm x 5 µm (Phenomenex). The mobile phase was a mixture of methanol, acetonitrile, and 50 mM oxalic acid (10: 20: 70%). The first wavelength was 354 nm, and the second one was 255 nm.

E. Calibration. Matrix matched standards (MMS) were used and spiked in sample at concentrations of 25, 50, 100, 150 and 200 µg kg-1.

F. Method validation. The method was validated in beef meat samples according to procedures described in Commission Decision 2002/657/EC [5]. The specificity, linearity, precision (repeatability and within-laboratory reproducibility), recovery (trueness), decision limit (CCα), and detection capability (CCβ) were evaluated. For validation purposes, blank samples were collected from farm animals not treated with TCs, and they were spiked with working solution at concentrations corresponding to 0.5xMRL, MRL and 1.5xMRL (50, 100 and 150 µg /kg).

Results

Validation parameters: repeatability, reproducibility, decision limit and detection capability were calculated from the results of analysis of 3 series (analyses in different occasions) of the control samples. Control samples of tissue were spiked with TCs on three concentration levels (50, 100, 150 µg/kg) and analyzed in six replicates. The concentration of each drug was determined and intra- and inter-assay coefficients of variations (CV) were calculated. Decision limits (CCα) and detection capabilities (CCβ) were calculated according to requirements of Commission Decision 2002/657/EC, using results of reproducibility analysis.

Table 1. Coefficients of TCs calibration curves and regression coefficients of the method.

	Slope	**Intercept**	**R^2**
OTC	0.7	2.8	0.9953
TC	1.2	-3.1	0.9955
CTC	1.1	2.3	0.998

Table 2. The intra-laboratory coefficients of variation CV (%), repeatability

	OTC (µg/kg)			**TC** (µg/kg)			**CTC** (µg/kg)		
	50	100	150	50	100	150	50	100	150
CV %	3.9	2.2	1.0	3.1	6.9	1.2	1.6	6.9	2.0

In that procedure the LOD is estimated as mean of 20 control sample assay results plus 3 times the standard deviation of the mean. The LOQ then becomes the mean of the same results plus 10 times the standard deviation of the mean [7]. Results showed that the detection limit of the tested drugs (TCs) was 25µg/kg and the limit of quantitation was found to be 50 µg/kg.

Linearity of the method of the response of TCs from tissue sample was determined by analysis of calibration curves which was generated running through HPLC analysis calibration standard solution in the concentration levels over the working range ((0-200 µg/kg), and plotting the recorded peak areas versus concentrations. Regression coefficients of curves indicated a good fit for all the analytes (r^2>0.995) (Table 1).

Repeatability determined at three concentration levels analyzed in 3 series of samples (n=6), (50, 100, 150 µg/kg), expressed as coefficient of variation CV %. The coefficients of variation are listed in Table 2

They all comply with the Horwitz-equation which states that for each fortification level the intra-laboratory CV should not exceed 15 % (50-66% 0f Horwitz reproducibility CVs).

Intra-laboratory reproducibility determined at three concentration levels analyzed in 3 series of sample, expressed as coefficient of variation % (Table 3).

The specificity was assessed studying the absence of any interference at the elution times of the analytes. For this purpose twenty blank tissue samples were analyzed. The adopted cleanup gives chromatograms free of interferences.

According of Commission Decision 2002/657/EC, CCα and CCβ replace the limit of detection and the limit of quantification, respectively. The values of the decision limit and detection capability are shown in Table 4.

Table 3. The intra-laboratory coefficients of variation CV (%), reproducibility

	OTC (µg/kg)			**TC** (µg/kg)			**CTC** (µg/kg)		
	50	100	150	50	100	150	50	100	150
CV %	4.6	6.6	7.2	4.5	5.9	5.3	2.9	7.6	6.9

Table 4. Results of decision limit CCα and detection capability CCβ for TCs.

Analite	**No. eplicates**	**Conc. (µg /kg)**	**Recovery (%)**	**RT (min)**	**CCα (µg /kg)**	**CCβ (µg/ kg)**
OTC	6	50	82.5	6.6		
	6	100	90.0		104.6	108.2
	6	150	96.8			
TC	6	50	87.2	8.9		
	6	100	89.7		119.8	127.4
	6	150	97.3			
CTC	6	50	87.8	12.2		
	6	100	98.1		113.2	122.7
	6	150	93.5			

The method was employed for monitoring of 37 beef muscle samples collected from different slaughterhouses of Tirana, during June 2012 to May 2013, in which only 4 samples showed detectable concentration of OTC residues but were lower than the maximum residue limits (MRLs) according to Commission Regulation (EU) No 37/2010. The residual concentrations of OTC muscles ranged from 57-100 µg/kg (Table 5). TC and CTC were absent in all samples.

Representative chromatogram obtained during the quantification of TCs in beef meat contaminated with OTC 67.2 µg/kg is shown in Figure 1.

Table 5. Level of antibiotic residues in beef muscle samples collected from different slaughterhouses of Tirana.

Analysis of Antibiotics Residues	**Level of Chemical Residues (µg/kg)**			**MRL**
	Slaughterhouse behind the sports palace "Asllan Rusi"	**Slaughterhouse KMY. Street "3 Deshmoret", Yzberisht**	**Slaughterhouse "Kthesa Koder Kamze"**	
OTC	57.4	82.9	67.2	**100**
			98.3	

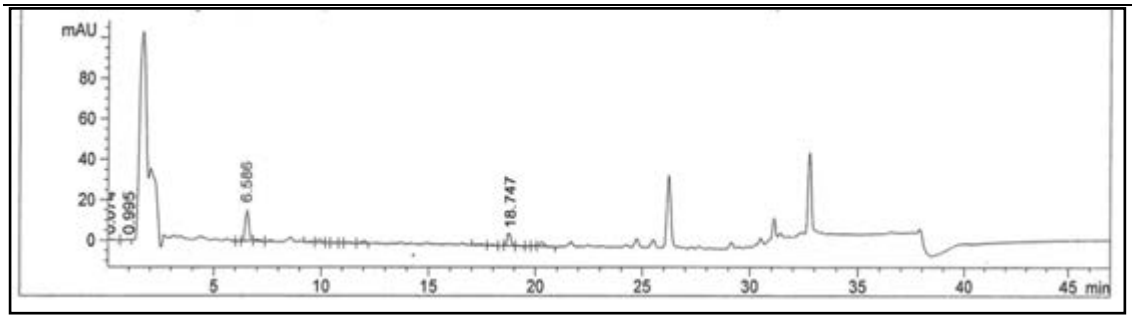

Figure 1. Representative chromatogram obtained during the quantification of TCs in beef meat contaminated with OTC 67.2 µg/kg. The retention time of OTC is 6.586 min.

Discussions

A simple, rapid and sensitive HPLC method for the monitoring of tissue residues of TCs in beef meat samples was modified and validated. The samples were analyzed by using High Performance Liquid Chromatography (HPLC), with Photo Diode Array detector (PDA). Regression coefficients of curves indicated a good fit for all the analytes (r^2>0.995).

Repeatability determined at three concentration levels analyzed in 3 series of samples (n=6), (50, 100, 150 µg/kg), expressed as coefficient of variation CV % did not exceed 15 %. Intra-laboratory reproducibility determined at three concentration levels analyzed in 3 series of sample, expressed as coefficient of variation % did not exceed 23 %.

Results showed that the detection limit of the tested drugs (TCs) was 25µg/kg and the limit of quantitation was found to be 50 µg/kg. The recoveries of OTC, TC and CTC from spiked samples at three fortification levels, were higher than 78% for all drugs.

The method was employed for monitoring of 37 beef muscle samples collected from different slaughterhouses of Tirana, during June 2012 to May 2013, in which only 4 samples showed detectable concentration of OTC residues but were lower than the maximum residue limits (MRLs) according to Commission Regulation (EU) No 37/2010. The residual concentrations of OTC muscles ranged from 57-100 µg/kg. TC and CTC were absent in all samples. The presence of this component in tissue samples might be due to slaughtering of animals without giving adequate withdrawal period of time.

Conclusions

The study made for the period from June 2012 to May 2013 to estimate the proportion of tetracycline residue levels in 37 beef meat samples collected from different slaughterhouses of Tirana, capital of Albania, only 4 samples showed detectable concentration of OTC residues. Their concentrates were lower than the maximum residue limits (MRLs) according to Commission Regulation (EU) No 37/2010. TC and CTC were absent in all samples. These levels were not able to induce risks to human health. However other studies are necessary to evaluate other drug residues in beef samples and to evaluate the hazards of these residues in relation with daily intakes and other related factors.

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