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Original scientific paper
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THE CONFIRMATION AND QUANTIFICATION OF TETRACYCLINE ANTIBIOTIC RESIDUES IN MILK BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

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Abstract

Tetracyclines are broad-spectrum antibiotics, so they are widely used to treat infection diseases in veterinary and human medicine. The inappropriate use of antibiotics can lead to the presence of residues in milk and this can pose a risk to health of humans. Therefore, the European Union has established maximum residue levels (MRLs) for most antibiotics in milk and animal tissues. The aim of this study is validation of LC-MS/MS method for detection of tetracyclin (TC) and oxytetrayclin (OTC) antibiotics in milk.
The samples were cleaned on an Oasis HLB solid-phase extraction cartridge. Chromatographic separation was carried out on C18 column (50 x 2.1 mm, 1.7µm) with mobile phase A: 0.1% formic acid in water and mobile phase B: 0.1% formic acid in acetonitrile. Validation of the method was performed according the criteria of Commission Decision 2002/657/EC. In validation procedure were evaluated: linearity, decision limit (CCα), detection capability (CCβ), accuracy, precision and reproducibility of the method.
The results for optimization of MS/MS method are with agreement with reference's data. The method demonstrated good linearity on the concentration range from 50.0 to 300.0 µg/L CCα and CCβ were below MRLs. The precision and reproducibility were evaluated by RSD, % (relative standard deviation,%). The recovery values were varies from 70-105% with RSD <20%.
In conclusion, the LC-MS/MS method was sensitive and suitable for routine analysis for detection of these antibiotics in milk.

Keywords: tetracyclin antibiotics, milk, validation, LC-MS/MS method.

INTRODUCTION

Tetracyclines (TCs) are broad spectrum antibiotics of antibacterial activity against most gram-positive and gram-negative bacteria, derivatives of the polycyclic naphthacene carboximide, that produced by streptomyces. The basic frame structure of tetracycline antibiotics included the tetracene, which can be widely used for the prevention and treatment of animal diseases, they were unstable in acidic and alkaline conditions, and could formatted insoluble chelate with a variety of metal ions under the neutral condition (M. Khaskheli *et al.* 2008, De Ruyck H *et al.* 2007, Avinash Dalmia 2016).
Mechanism of action of tetracycline antibiotics was by binding reversibly to the 30S subunit of the bacterial ribosome and preventing the aminoacyl tRNA from binding to the A site of the ribosome. They also bind to some extent the bacterial 50S ribosomal subunit and may alter the cytoplasmic membrane causing intracellular components to leak from bacterial cells. This inhibits addition of amino acid to the growing peptide resulting in inhibition of protein synthesis. (Fig. 1) (Hu Yu *et al.* 2012)

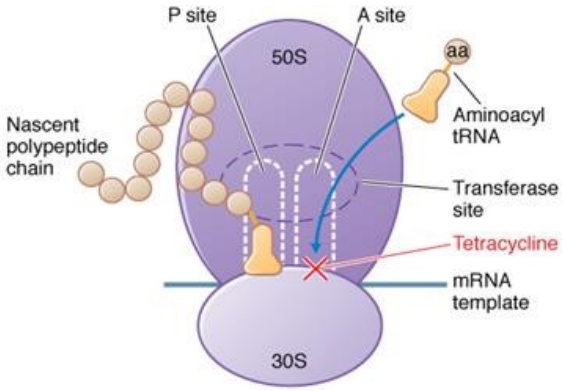
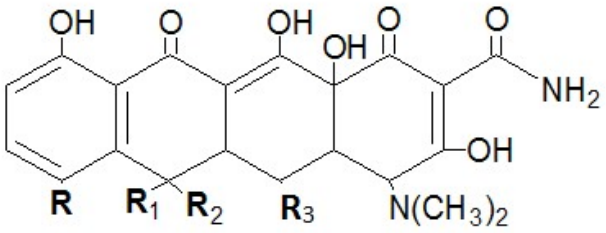


Figure 1. Mechanism of action of tetracycline antibiotics

The antibacterial activity, as well as physicochemical and pharmacokinetic properties are strongly related to TCs chemical structure (Figure. 2) (Mei Biea et al. 2012).



Compound	R	R1	R2	R3
Tetracycline	-H	- CH3	-H	-H
Oxytetracycline	-H	- CH3	-OH	-OH

Figure 2. Chemical structure of tetracycline antibiotics

The inappropriate use of antibiotics can lead to the presence of residues in milk and this can pose a risk to health of humans. These antibiotic residues in food can cause toxicity and side effects such as allergic reactions, rash and nausea, etc. In addition, the low levels of antibiotics in food products, consumed for long time, can lead to the spread of drug-resistant bacteria.
Tetracyclines can damage liver and kidney and influence the growth of skeleton and can enhance the drug-resistance of bacteria(Hu Yu et al. 2012).
Therefore, the European Union has established maximum residue levels (MRLs) for most antibiotics in milk and animal tissues. In the EU, the maximum residue limit (MRL) for antibiotics is established according to (EU) 37/2010. Decision 2002/657/EC also establishes the method for determination of these compounds in milk and animal tissues (Commission Regulation (EU) 37/2010, Commission decision: 2002/657/EC).
According with these requirements, the Republic of North Macedonian legislation was fully aligned with the EU legislation concerning residues of antibiotics in foodstuffs of animal origin in regarding the Council regulation 37/2010/EU (Official Gazette of Republic of North Macedonia 80/2011).
Recently, the basic advances in developing sensitivity and specifity of food analyses of pharmaceutical residues are due to the technology instrument, the application of liquid

chromatography with tandem mass spectrometric detection (LC–MS/MS), which is more sophisticated technique allows a very effective isolation of analyte ions from the noise-producing matrix. The LC–MS/MS produces a high speed of analysis, greater resolution, higher peak capacity and sensitivity (Swartz et al. 2005; Tylová 2010).
The aim of this study is validation of LC-MS/MS method for detection of tetracyclin (TC) and oxytetrayclin (OTC) antibiotics in milk.

MATERIAL AND METHODS

Reagents. All organic solvents were HPLC grade and all chemicals were analytical grade. Methanol, acetonitrile, water, were purchased from Carlo Erba, formic acid, dimethyl sulfoxide (DMSO), ammonium hydroxide, sodium chloride, trichloroacetic acid, citric acid monohydrate, disodium hydrogen phosphate dihydrate and disodium salt of ethylenediaminetetraacetic acid, were of analytical grade (Sigma-Aldrich).
Analytical standards and standard solutions. Tetracyclin (TC) and Oxytetrayclin (OTC), were obtained from Sigma-Aldrich Chemical Company (USA). Stock standard solutions of TCs (1 mg/mL) were prepared by weighing 10.0 ± 0.1 mg of each substance and quantitative transfer to a 10 mL volumetric flask and filling to volume with methanol.
Apparatus: The LC-MS/MS system was purchased from Waters. The LC system equipped with binary pump, vacuum degasser, thermostated autosampler and thermostated column manager. The chromatographic separation was achieved on a Kinetex®C18 (1.7µM100A, LC Column 50x2.1 mm) column followed by tandem mass spectrometry using an electro spray ionization source in positive mode.
For solid phase extraction were used OASIS® HLB 3cc (60 mg) extraction cartridges by Waters (Ireland).

Sample preparation

a) Transfer 5 ml of milk in 50 ml of tube
b) 2 mL of 20% trichloroacetic acid
c) Shaking 15 min
d) 20 mL of McIlvaine buffer
d) Centrifuge for 15 minutes at 4000 rpm, at + 4ºC
e) SPE HLB Oasis cartridge
f) activated with 3 mL of methanol and 2 mL of water
g) washed with 4 mL of water
f) eluted with 3 mL of methanol
h) Dry in N2 flow and dissolve the residue with 500 µl mobile phase.
i) filtered on a 0.22 µm micro filter injected into LC-MS/MS system

LC-MS/MS Conditions

The mobile phase A: aqueous solution with 0.1% of formic acid (0.1% HCOOH / H2O), and mobile phase B was acetonitrile with 0.1% of formic acid (0.1% HCOOH / CH3CN)). The best results were observed at 40 °C, 0.4 mL/min as the flow rate, (Tomáš Šopík *et al.* 2012), they are given in Table 1.

Table 1. Phase and flow ratios

Time (min)	Flow(ml/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0,4	98.0	2.0
0,75	0,4	98.0	2.0
7,0	0,4	50.0	50.0
11.0	0,4	0.00	100.0
11.5	0,4	98.0	2.0
13.0	0.4	98,0	2.0

In order to determine the retention time and the mass spectrum of antibiotics involved in this study, individual standards of these analytes with a concentration of 10 µg / mL were directly injected into the mass detector, in the ESI + (positive ionic spectrum) and were determined main precursor ions, product ions (daughter ions) (Andreia Freitas et al. 2012, Anderson, C. R et al. 2005). The MS / MS conditions are given in Table 2.

Table 2. The MS / MS conditions

Type of ionization	ES+	Desolvatation gas flow (L/Hr)	500
Capillary (kV)	4.0	LM 1 resolution	11
Cone (V)	26	HM 1 resolution	14.7
Extractor (V)	3.0	Ion energy 1	0.5
RF Lens (V)	0.1	Entrance	50
Source temperature °C	150	LM 2 resolution	10.0

VALIDATION PROCEDURE

The method was validated using the regulatory guidelines from the Commission Decision 2002/657/EC, concerning the performance of analytical methods.
Specificity was determined by analyzing 20 blank bovine milk samples for the verification of interference, using the optimized extraction procedure and chromatographic conditions described above. The blank bovine milk were obtained from untreated cattle.
The linearity of the method was determined by the coefficient of correlation (R^2) from the calibration curves for each components. The correlation coefficient was evaluated. For the calibration curve, the linear regression equation ($y = ax + b$) was applied.
For determination of CCα and CCβ the milk was enriched with the standards below the MRL value, and during this were prepared 18 replicates. The CCa equals the corresponding concentration at the intercept plus 2.33 times the standard deviation of the intra-laboratory reproducibility of the intercept. CCβ was calculated as the decision limit CCα plus 1.64 times the corresponding standard deviation (β = 5%), supposing that standard deviation at the MRL is similar to that obtained at the CCα level. The percentage recovery was evaluated in the same experiment as repeatability by comparing the mean measured concentration with the fortified concentration of the samples.

RESULTS AND DISCUSSION

Optimization of the MS / MS method and the standards injection into the MS detector was achieved ionic scanning and analyzed parameters were determined such as: precursor ions, daughter ions and retention time) that are presented in Table 3.
The results obtained for precursor ions, daughter ions, retention time correspond to the literature data by (Andreia Freitas et al. 2012., Susan B. Clark1 e al 2012, Rameshwari Amatya et al. 2010)

Table 3. Retention time, precursor and daughters ions

Compound	Formula/Mass		Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode	Retention time
Tetracyclin	$444.4+H^+=445$	1 2	445.05 445.05	26 26	410.08 97.92	20 48	ES+	3.29
Oxytetracyclin	$460.4+H^+=461.4$	1 2	462.01 462.01	46 46	97.92 153.98	38 30	ES+	4.23

The linear regression analysis showed good correlation with R^2 from 0.9881 for Tetracyclin and 0.9801 for Oxytetracyclin. Linearity of the method was performed according to Decision 2002/657/EC.

Table 4. Linearity of the method

Compound	R^2
Tetracyclin	0,9881
Oxytetracyclin	0,9801

From the results shown in Table 5 it can be concluded the obtained value for CCα and CCβ was less than MRLs (Maximum Residue Limits).

Table 5. Validation parameters for the method

Compound	Level of Spike ng / ml	Recovery	CCα (ng/ml)	CCβ (ng/ml)	RSDr
Tetracyclin	50	74.16%	10.5	11.25	9.44%
Oxytetracyclin	50	85.36%	9.59	10.2	7.54%

The CCα ranged from 9.59 ng/mL to 10.5 ng/mL, and the CCβ ranged from 10.2 ng/mL to 11.25 ng/mL. It is concluded that the methods showed relevant CCα and CCβ according to the 2002/657/EC. The recoveries were in the range between 74.16% and 85.36%.
The results obtained for Validation parameters for the method correspond to the literature data by Tomáš Šopík et al. 2012, Pailler, et al. 2009, Chopra, I et al. 2001, Ewelina Patyra et al. 2014.

CONCLUSION

The confirmatory methods are more accurate, more sensitive, more precise than screening methods and enable the identification and quantification of analytes. According to 2002/657/EC "confirmatory method provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest", while "screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest". The results of this work show that SPE and LC-MS/MS method is a robust, sensitive, and repeatable instrument for the study of tetracyclines residues in a milk matrix. The method can be used for routine analysis of milk samples.

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