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ANALYTICAL PROCEDURE FOR THE DETERMINATION OF LINCOMYCIN AND TYLOSIN ANTIBIOTIC RESIDUES IN MILK BY LIQUID CHROMATOGRAPHY-COUPLED TANDEM MASS SPECTROMETRY (LC–MS/MS)

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Abstract

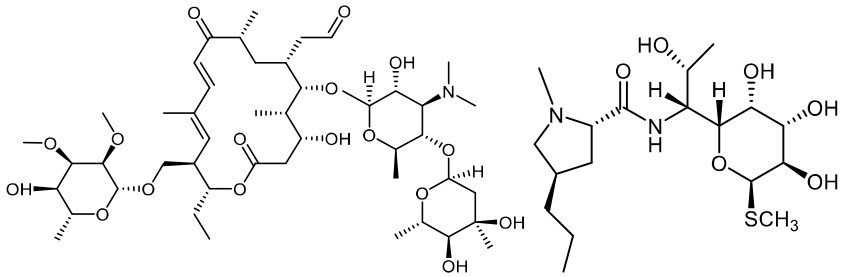
A rapid and sensitive liquid chromatography / mass spectrometry (LC–MS/MS) method has been developed and validated for the simultaneous identification, confirmation, and quantitation of tylosin and lincomycin residues in milk. The method was validated in accordance with Commission Decision 2002/657/EC. McIlvaine buffer was used to extract residues from milk, and further SPE cleanup was performed using an Oasis HLB cartridges. Chromatographic separation was achieved using a 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. The linear regression analysis showed a good correlation with R^2 from 0.9879to 0.9978. The recoveries of target compounds were 84.5 % for lincomycin and 92.70 % for tylosin. The decision limits (CCα) for lincomycin was 15.30µg/L and for tylosin 13.50 µg/L. Detection capability (CCβ) was 17.60 µg/L and 16.85 µg/L, respectively. From the validation study results can conclude that the method is suitable to be applied for routine analysis of the residues of lincomycin and tylosin in milk.

***Keywords**: lincomycin, tylosin, milk, validation, LC-MS/MS method*

1. Introduction

Macrolides and lincosamides are two classes of antibiotic with similar antibacterial activity, but differing in chemical structure. They are a group of veterinary antibiotics widely used to treat, prevent diseases and promote growth in food-producing animals [1].

Tylosin is a broad spectrum macrolide-class antibiotic, constituted by a macrocyclic lactone formed by 16 carbons linked to sugar molecules through glycosidic bonds, produced by a strain of *Streptomyces fradiae* (Figure 1) [2].



a) b)

Figure 1. The chemical structure: a) Tylosin and b) Lincomycin

The lincosamide are broad -spectrum antibiotics consist of lincomycin. The structure of lincomycin itself is of a five-membered cyclic amino amide in attached to a thioglycoside side-chain and is produced by *Streptomyces lincolnensis* (Figure 1). They are bacteriostatic compounds that reversibly bind to the 23S rRNA in the 50S ribosome subunit and inhibit mRNA-directed protein synthesis and thus, cell growth of susceptible bacteria [3].

Inappropriate or abusive use of antibiotics in farm animals might provoke their residues in food of animal origin and their metabolites may cause damage to the human vestibule and cochlear nerves, even to liver and kidneys, allergic reactions, toxic effects and may lead to an increase in human resistant strains [4,5].

The use of veterinary drugs is regulated through EU Council Regulation 37/2010 [6], which describes the procedure for establishing MRLs for veterinary medicinal products in foodstuffs of animal origin. MRLs values for antibiotics in milk, as set by the European Union, are summarized in Table 4. Council Directive 96/23/EC [7], contains guidelines for controlling veterinary drug residues in animals and their products with detailed procedures. Technical guidelines and performance criteria (detection level, selectivity, and specificity) for residue control in the framework of Directive 96/23/EC are described in Commission Decision 2002/657/EC [8] concerning the performance of analytical methods for the determination of organic residues and contaminants in living animals and animal products. According to these requirements, the Macedonian legislation was fully aligned with the EU legislation concerning residues of antibiotics in foodstuffs of animal origin regarding the Council regulation 37/2010/EU [9]. Therefore, sensitive and reliable analytical methods for the determination of veterinary residues in food of animal origin are needed to ensure consumers' safety.

Screening methods are usually inexpensive and rapid for analysis but do not provide unequivocal confirmative/quantitative results [10-12]. Confirmatory methods are methods that provide full information for simultaneous analysis of antibiotic residues of different classes and quantification at the level of interest. Liquid chromatography tandem mass spectrometry (LC–MS/MS) has become the technique of choice for multiclass analysis of drug residues, the latter allowing increased sensitivity and selectivity in milk [13-16]. Many studies have reported methodologies based on liquid chromatography tandem mass spectrometry (LC–MS/MS) to determine macrolides and lincosamides residues in milk [17-21].

The aim of this study was to develop and validate of LC-MS/MS method for the determination of tylosin and lincomycin antibiotic residues from different classes: macrolides and lincosamide, in bovine milk, applying thereby the analytical criteria stipulated under the Commission Decision 2002/657/EC.

2. Material and Methods

2.1. Analytical standards, chemicals and reagents:

Standards: Tylosin and Lincomycin were supplied by Fluka-Vetranal.

Methanol (LC-MS grade), acetonitrile (LC-MS grade), water (LC-MS grade), trichloroacetic acid, disodium hydrogen phosphate dihydrate and disodium salt of ethylenediaminetetraacetic acid were purchased from Carlo Erba, formic acid, dimethyl sulfoxide (DMSO), ammonium hydroxide, sodium chloride, citric acid monohydrate, formic acid was of analytical grade from Sigma-Aldrich.

2.2. Standard solutions

For all substances, individual stock standard solutions of 1mgmL^{-1} were prepared in methanol (MeOH) and kept at –20 °C. Mixtures of working standard solution from 1, 10 and 100 ngmL^{-1} of all antibiotics were prepared in the mobile phase. Calibration (working) mixed standard solutions with concentration levels 10, 25, 50, 75, 100, and 150.00 ng/mL were obtained from the intermediate solution at 1, 10 and 100 ng/mL.

2.3. Sample Preparation and Extraction Procedure

The volume of 2 mL 20% trichloroacetic acid in water was added on aliquot of 5 mL of milk and shaken for 5 min. After shaking, 20 mL of McIlvaine buffer was added. The samples were vortexed for 1 minute and centrifuged at 4000 rpm for 20 min at +4 °C. The supernatant was immediately applied to SPE HLB Oasis cartridge, previously activated with 3 mL of methanol and 2 mL of water. The column was washed with 4 mL of water. Antibiotic residues were eluted with 3 mL of methanol. The samples were evaporated to dryness under a stream of nitrogen at 35 °C. The dry residue was reconstituted with 250 μL of the mobile phase and filtered on a 0.22 μm microfilter. The volume of 10 μL of the final extract was injected into LC-MS/MS system.

2.4. LC-MS/MS Analysis

The LC-MS/MS system was purchased from Waters. The LC system equipped with a binary pump, vacuum degasser, thermostated autosampler, and thermostated column manager. A triple quadripole mass detector set in a positive ESI mode was used for the determination of the targeted antibiotics. For separation of antibiotics were used Kinetex® C18 column (1.7 μM 100 A, LC Column 50 x 2.1 mm). MassLynx software version 4.1 was used for data acquisition and calculation of results. The optimum conditions of selective reaction monitoring (SRM) were carried out at the following parameters: source temperature was set at 150°C, capillary voltage of 4.0 kV, nitrogen as desolvation gas at a flow rate of 500 L/h, nitrogen as nebulizer gas at a flow rate of 100L/h, desolvation

temperature was 400 °C. The values of collision energy, transitions for the SRM mode, are given in Table 1.

Table 1. The MS / MS conditions

Type of ionization	ES+	Desolvatation gas flow (L/Hr)	400
Capilary (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

2.5. Chromatographic Condition

Analytes were separated chromatographically using Kinetex®C18 (1.7μM100A, LC Column 50x2.1 mm) column at 35 °C with an injection volume of 10 μL. The flow rate was 0.4 $mL \cdot min^{-1}$. The total run time for each injection was 13 min. The mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitril with 0.1% formic acid. Elution gradient program is given in Table2.

Table 2. The liquid chromatography gradient elution method

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

2.6. Validation Procedure

The method was validated using the regulatory guidelines from the Commission Decision 2002/657/EC, concerning the performance of analytical methods. According to those requirements, specificity, recovery, repeatability, reproducibility, decision limit (CCα) and detection capability (CCβ), were determined.

Method linearity was evaluated by performing calibration curves. Calibration curves were constructed by least-squares linear regression analysis of the peak area, and linearity was determined by the coefficient of correlation (R^2). 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 was obtained from untreated cattle. For determination of CCα and CCβ the milk was enriched with the standards below the MRL value. The precision of this method was demonstrated in term of repeatability (intra-day precision) and reproducibility (inter-day precision). The precion was expressed with a coefficient of variation (CV, %). The accuracy of the method was assessed by recovery test.

3. Results and Discussion

In this study was used a wide range of analytes with different physicochemical properties. Because the bovine milk contains interferences such as proteins, lipide and lactose the success of the extraction procedure depended on the effective degreasing and deproteinization. The extraction with solution of TCA, compounds from all tested classes of antibiotics were isolated with variable recovery [19-21]. Mcllvain buffer were found to be effective to extract antibiotic residues from milk because they reduced co-extraction of non-polar matrix components (e.g. lipids) [22] and further SPE cleanup was performed using an Oasis HLB cartridges [15,18,19]. The method development started with the optimization of the MS parameters for the detection of the selected drugs. For determination of parent ion, a full-scan spectrum of each substance was collected in scan mode for the mass range m/z 50-1000. For all substances protonated, [MH]+ ions were detected with the highest abundance, using these precursor ions to obtain at least two product ions for each analyte. The mass spectrometer was operated in electro spray positive ionization mode (ESI+) [17-21] that are presented in Table 3.

Table 3. Retention time, precursor and daughters ions

Compound	Formula/Mass		Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode	Retention time
Tylosin	916.1+ H^+=91 7.1	1 2	916.43 916.43	56 56	174.07 100.97	46 56	ES+	6.31
Lincomycin	406.5+ H^+=40 7.5	1 2	407.09 407.09	34 34	126.02 41.79	30 72	ES+	2.59

Linearity of the method was performed according to Decision 2002/657/EC. The response of the detector was linear for each target compounds in the wide range with correlation coefficient (R^2) from 0.987 for Lincomycin and 0.9991 for Tylosin. The CCβ values ranged from16.85 μg/ L, for Tylosin to 17.60 μg/L for Lincomycin, while the CCα values ranged from 13.50 μg/ L for Tylosin to

15.30μg/ L for Lincomycin. In other words, analytes were correctly detected in bovine milk and the obtained values for CCα and CCβ were below MRL. The results are presented in Table 4.

Table 4. CCα, CCβ, MRL for antibiotics in bovine milk, linearity of the method (R^2)

No.	Antibiotic	CCα μg/ L	CCβ μg/ L	R^2	MRL μg/L
1	Tylosin	13.50	16.85	0.999 1	50
2	Lincomycin	15.30	17.60	0.987 7	100

None significant interference has been observed, the method was considered as specific for the target compounds. CV values for all examined antibiotic agents were lower than 15.54%, while mean apparent recovery rates for all target compounds ranged from 84.53% to 92.70%. The values are very satisfactory and confirmed good method performance quite suitable for the control of milk samples for the majority of compounds. The results are presented in Table 5.

All these validation parameters indicate the good performance of the proposed analytical method. The results obtained for validation parameters for the method correspond to the literature data by Cristiana B.et al 2006, Bladek T. et al. 2010, Marilena E. D et al. 2015, Zhang Y. et al. 2015, Gaugain-Juhel M. et al. 2009.

Table 5. Validation parameters

No.	Antibiotic	Level of spike ng/mL	Recovery %	RSD_r %
1	Tylosin	92.70	92.70	11.44
2	Lincomycin	84.50	84.53	15.54

4. Conclusion

Monitoring of antibiotics residues is necessary to ensure food safety and to prevent exposure of the consumers. A precise and simplified LC-MS/MS method for identification and quantification of the antibiotics tylosin and lincomycin in bovine milk was developed. The method was successfully validated according to the European Union requirements. The method will be used for routine control of veterinary drugs in milk.

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