RAPID RP-HPLC METHOD WITH PDA DETECTION FOR TYLOSIN DETERMINATION IN LIQUID SAMPLES

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ABSTRACT

As one of the widely used veterinary macrolide antibiotics, the environmental risks of tylosin (TYL) received more and more attention. The purpose of the present study was to develop a modified, rapid and precise RP-HPLC method with PDA detection for the determination of the macrolide antibiotic tylosin in liquid samples. The proposed two HPLC methods offered short retention time (< 6.8 min), high precision ($RSD \leq 1.40\%$) and linearity ($R^2 > 0.9993$). HPLC chromatograms exhibited well-resolved peaks with no interference peaks. Despite of the higher linearity (R^2 0.9998) of the applied HPLC Method I, the lower LOQ and LOD values of HPLC Method II indicated its better suitability for detection and quantification of low tylosin concentrations in aqueous medium ($>2.78 \mu\text{g/mL}$).

Key words: tylosin, RP-HPLC, photodiode array (PDA) detector, liquid samples

INTRODUCTION

Antibiotics present in the environment are recently considered as „*emerging*” contaminants, and have raised increasing concerns about their potential risks to ecosystems and human health.

In European Union countries, it was estimated that approximately 4700 t of antibiotics were used in veterinary medicine (1). In Germany, the consumption of veterinary antibiotics was estimated to 1700 t per year from 2011 (2).

Tylosin (TYL) belongs to the group of broad-spectrum macrolide antibiotics used widely in veterinary medicine as anticoccidial feed additives in poultry and livestock, as growth promoters, for improved feed efficiency in ruminants and due to their antibiotic activities against Gram-positive microorganisms (3, 4).

However, a portion of the administered dose used for these purposes can be excreted into animal manure, and land application of the animal manure as plant fertilizers enhances the dissemination of antibiotics in the environment following the pathway: accumulation in the top soil layers – leaching to groundwater – subsequent transport to surface waters (5, 6).

Thus, quantitative analyses of macrolide antibiotics in water is essential to evaluate their environmental exposure and subsequent risks to environmental quality and public health (1). Sokol et al. (2010) developed a HPLC method combined with solid phase extraction (SPE) for the determination of TYL residues in the food of animal origin (7). Pietron et al., (2011) presented an analytical procedure for the determination of tylosin A in feedingstuff by liquid chromatography-ultraviolet detection (8). Ahmed et al., (2013) determined three macrolide antibiotics: tylosin (TYL), spiramycin (SPIR), and erythromycin (ERY) in buffaloes' meat using a modified method of thin-layer chromatography–bioautography (TLC–B) (9). Another method successfully applied for tylosin

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tartrate detection in aqueous phase was UV-VIS spectroscopy (10). The study of Hoese *et al.*, (2009) assessed the runoff potential of tylosin and chlortetracycline (CTC) from soils treated with manure from swine fed rations containing the highest labeled rate of each chemical. The concentrations of tylosin were determined by LC/MS analysis (11).

However, only a limited number of studies have thus far focused on the detection of TYL in aqueous phase by HPLC, which provoked the current investigations.

The aim of the present study was to develop a modified, rapid and precise RP-HPLC-PDA method for TYL determination in aqueous samples.

MATERIALS AND METHODS

Chemicals

Tylosin ($C_{46}H_{77}NO_{17}$), acetonitrile (ACN, $\geq 99.8\%$), orthophosphoric acid (H_3PO_4 85%), methanol ($\geq 99.9\%$), ethanol (p.a. $\geq 99.8\%$), and $Na_2HPO_4 \cdot 2H_2O$ (HPLC, $\geq 98.5\%$) HPLC grade, were obtained from Sigma-Aldrich.

HPLC system and conditions

RP-HPLC system comprising of a Hypersil BDS C_{18} (5 μ M, 4.6 x 150 mm) column, Surveyor LC Pump Plus, PDA detector, and Surveyor Autosampler Plus (Thermo Fisher Scientific) was used. The mobile phase consisted of a mixture of ACN and 0.1M H_3PO_4 (60:40, v/v). The buffer pH was adjusted to 2.5 with H_3PO_4 .

The samples were monitored at 290 nm. All analyses were made in triplicate. Standard stock solutions (100 μ g/mL) were prepared by dissolving the appropriate amount of TYL in ethanol. Working standard solutions (5 – 100 μ g/mL) were prepared daily by dilution with Milli-Q water. The volume injected into the HPLC column was 20 μ L.

All HPLC analyses were made in triplicate. The experimental data was analyzed by regression analyses and determination of the corresponding correlation coefficients (R^2), standard error of the intercept (SE_y), standard error of the slope (SE_x) and the relative standard deviation (RSD, %).

The efficiency and accuracy of the developed HPLC method was estimated based on the calculated limit of detection (LOD) and limit of quantification (LOQ).

Statistical analyses

The statistical significance of the experimental results and the values of SE_y , SE_x , RSD (%), LOD (μ g/mL) and LOQ, μ g/mL were determined by XLSTAT Statistical Software for Excel.

RESULTS AND DISCUSSION

Physicochemical characteristics of TYL

The software package CS Chem 3D ultra was used to calculate the basic molecular characteristic of TYL. The molecular formula of the compound is presented in **Figure 1**.

Figure 1. Structural (A) and 3D (B) molecular formula of TYL.

The physicochemical and molecular properties of TYL are displayed in **Table 1**.

The development and optimization of a sensitive and rapid HPLC method for TYL determination

in aqueous phase included the performance of several preliminary experiments testing different HPLC conditions: mobile phases, temperatures and wavelengths.

Table 1. Physicochemical properties and molecular characteristics of TYL.

Molecular formula	C ₄₆ H ₇₇ NO ₁₇
CAS No.	1401-69-0
UIPAC name	[(2R,3R,4E,6E,9R,11R,12S,13S,14R)-12- {[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl- α -L-ribo-hexopyranosyl)-3-(dimethylamino)- β -D-glucopyrano-syl]oxy}-2-ethyl-14-hydroxy-5,9,13-trimethyl-8, 16-dioxo-11-(2-oxoethyl)oxacyclohexadeca-4,6-dien-3-yl]methyl 6-deoxy-2,3-di-O-methyl- β -D-allopyranoside
Trade names	Tylocine, Tylan
Molecular mass	916.10 g/mol
Solubility	soluble in lower alcohols, esters, ketones, chlorinated hydrocarbons, benzene, ether
pKa (12)	7.73
UV_{max} (12)	$\lambda \sim 282$ nm
Topological polar surface area (12)	239 Å ²
Connolly Accessible Area	1078.23 Å ²
Connolly Molecular Area	660.07 Å ²
Ovality	1.619

HPLC method I

The first experimental series were conducted with eleven TYL standard solutions with concentrations 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μ g/mL. The used mobile phase consisted of ACN and 0.1M H₃PO₄ (50:50, v/v). Optimal performance was obtained at a flowrate 0.8 mL/min. The effect of temperature on the separation process was studied in the range 18 –

30°C. Satisfactory peak resolution and optimum analyses time were established at 30°C at a wavelength λ 265 nm. TYL was successfully detected within 6.8 min. The obtained HPLC chromatograms did not contain interference peaks, which could influence the quantitative results (**Figure 2**), although a wide peak with small area was detected around 3.8 min.

Figure 2. HPLC chromatogram of TYL (C₀ 10 μ g/mL) – HPLC Method I.

The standard calibration curve based on the spectral peak areas is presented in **Figure 3**.

Figure 3. Standard calibration curve of TYL – HPLC Method I.

It is characterized with a high correlation coefficient R^2 0.9993 and the following linear equation (1):

$$y = 18207 \cdot x - 71417 \quad (1)$$

HPLC method II

The second experimental series were conducted with eleven TYL standard solutions with concentrations 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 $\mu\text{g/mL}$. The used mobile phase consisted of ACN and 0.1M H_3PO_4 (60:40, v/v).

Optimal performance was obtained at a flowrate 0.8 mL/min. The effect of temperature on the separation process was studied in the range 18 - 30°C. Satisfactory peak resolution and optimum analyses time were established at 30°C at a wavelength λ 290 nm. TYL was successfully detected within 5.4 min (**Figure 4**). The obtained HPLC chromatograms did not contain interference peaks, which could influence the quantitative results. Besides, the wide peaks registered on the chromatograms of HPLC Method I (**Figure 2**) are missing.

Figure 4. HPLC chromatogram of TYL (C_0 10 $\mu\text{g/mL}$) – HPLC Method II.

The standard calibration curve plotted based on the spectral peak areas is presented in **Figure 5**.

Figure 5. Standard calibration curve of TYL – HPLC Method II.

It is characterized with a higher correlation coefficient R^2 0.9995, as compared to HPLC Method I, and the following linear equation (2):

$$y = 22357.x - 31775 \quad (2)$$

Accuracy and sensitivity of the applied HPLC methods

The sensitivity of both methods was examined through measuring the limit of detection (LOD) and the limit of quantitation (LOQ) for TYL at a

signal-to-noise ratio of 3 and 10, respectively, according to the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) based on the standard deviation of the intercept (SE_y) and of the slope (SE_x) of the calibration curve of the analyte (13). The relative standard deviation (RSD, %) values of both HPLC methods were also calculated. The values of these parameters are presented in **Table 2**.

Table 2. Values of SE_y , SE_x , RSD, LOD and LOQ for both applied HPLC methods

Method	SE_y	SE_x	RSD, %	LOD, µg/mL	LOQ, µg/mL
HPLC Method I	8823.498	144.55	1.40	1.59	4.83
HPLC Method II	6234.161	102.13	0.89	0.92	2.78

Although, the regression analysis of both methods confirmed that the deviation of the y-intercept from zero for both calibration curves (**Figures 3, 5**) was not significant, the values of SE_x and SE_y of HPLC Method II were significantly lower (**Table 2**). The RSD values of both methods are acceptable as they are less than 10 % (14). Regarding the developed HPLC methodologies, and based on the data from Table 2, it could be concluded that undoubtedly Method II displayed the highest accuracy and efficiency as the determined RSD, LOD and LOQ values were the lowest. The comparative

estimation of the HPLC spectral data of TYL obtained by both liquid-chromatographic methods (**Figures 2, 4**) revealed that the spectral peaks of Method II characterized with 1.2 times greater area. Besides, they were significantly more pronounced in the entire tested concentration range. Another advantage of HPLC Method II is the registered shorter retention time – 5.4 min.

The latter conclusions prove the applicability of HPLC Method II for the determination of TYL content in real samples of wastewater effluents from veterinary clinics

and/or animal farms, which could enhance the control and prevention of water contamination with this veterinary antibiotic.

CONCLUSIONS

A RP-HPLC-PDA analytical methodology appropriate for the determination of the veterinary antibiotic TYL in aqueous phase was developed in the present study. Desirable chromatographic separation was achieved on a C₁₈ column employing a mixture of ACN and 0.1M H₃PO₄ (60:40; v/v) as the mobile phase. The obtained HPLC chromatograms were well pronounced and did not contain any interference peaks, which could influence the quantitative results. The applied method offered short analysis time (5.4 min), high precision (RSD 0.89 %) and high linearity (R² 0.9995). It characterized with satisfactory LOD (1.59 µg/mL) and LOQ (2.78 µg/mL) values. The simple and rapid method developed enhances the capabilities for the accurate and selective determination of low TYL concentrations in aqueous phase.

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