ANALYTICAL PROCEDURE FOR THE DETERMINATION OF TYLOSIN A IN FEEDINGSTUFF BY LIQUID CHROMATOGRAPHY–ULTRAVIOLET DETECTION

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Abstract

A reliable and selective liquid chromatography–ultraviolet detection method for determination of tylosin has been developed. The extraction of analyte from feedingstuffs was performed with solution of citric buffer and methanol. The extracts were cleaned up by solid phase extraction procedure using an octadecyl cartridge. Samples were brought up to dryness and dissolved in phosphate buffer. The analysis was carried out on C18 analytical column with UV detection at \( \lambda = 282 \text{ nm} \). The analytical procedure has been successfully adopted and validated for quantitative determination of tylosin A in feedingstuff samples. The validation included determination of specificity, linearity, repeatability, and within-laboratory reproducibility. Mean recovery for spiked samples was 84.7% within the working range of 5–1,000 mg/kg. The inter-day relative standard deviation was below 6.6%. The results of validation procedure proved that presented method is efficient, precise, and useful for routine analysis for screening of quality, homogeneity, and stability of medicated feedingstuffs.

Key words: feedingstuffs, tylosin, HPLC-UV method.

Macrolides is a group of important pharmacological agents with antimicrobial activity, which are mainly effective against Gram-positive bacteria. They inhibit protein synthesis of the microorganism by binding to 50S subunit of the ribosome. Macrolides in general do not bind to mammalian ribosomes, which makes them a relatively safe group of drugs for veterinary use (16). They are classified according to the number of lactone ring atoms (12 to 16 atoms) and seven of them are approved for use in food-producing animals (6). One of them - tylosin (Fig. 1) is 16-membered ring macrolide and pharmaceutically is defined as a mixture of four homologue substances: tylosin A - main component (>80%), tylosin B (desmycosin), tylosin C (macrocin), and tylosin D (relomycin). The drug has been used in veterinary treatment of: "pinkeye" in cattle, respiratory tract infection, swine dysentery, and other infections in many animal species. Tylosin was used for growth promotion in animal feeds until its withdrawal in the EU in 1998 (5), and currently only therapeutic treatment is available. In many countries medicated feedingstuffs are a main route of administration of tylosin to swine and poultry (6, 16).

Official control of medicated feedingstuff quality in Poland (4, 7, 15) revealed that more than 10% samples in 2007 and 42% in 2008 did not keep declared tylosin activity or homogeneity. Microbiological plate methods are used for monitoring purposes of feedingstuffs, but specificity of these methods is not satisfactory enough because several antibiotics can inhibit the growth of the bacterial colony. Therefore microbiological methods are not recommended for analysis of samples with complex chemical matrix (1, 2). The problem of analytical specificity may be solved by using instrumental chromatographic methods. Furthermore, quantification level of this type of methods is significantly better than other techniques. Some studies concerning instrumental determination of tylosin in medicated feedingstuffs were reported. They include liquid chromatography coupled with UV (13), DAD (9, 12), ECD (11), and MS-MS detection (8, 10, 18, 19).

The need for official control of homogeneity and stability of tylosin in medicated feedingstuffs (4) resulted in the development of an instrumental method of its quantification. The presented paper reports the results of optimisation of a simple and selective LC-UV method for the determination of tylosin A in medicated feedingstuffs.
Material and Methods

Reagents and chemicals. Tylosin tartrate standard was obtained from Sigma-Aldrich (USA). Acetonitrile, methanol, and orthophosphoric acid were purchased in POCh Gliwice (Poland), sodium hydroxide, citric acid monohydrate, and ammonium carbonate were obtained from Sigma-Aldrich (USA). N-hexane and C18 SPE cartridge (500 mg, 3 mL) were delivered by J.T. Baker (Germany). All chemicals were HPLC or analytical grade. Water was purified by the Milli-Q system.

Standard solution. Standard stock solution of tylosin A (1 mg/mL) in methanol was prepared and stored in freezer up to 3 months (-18°C) (14). Correction for the type of salt used, purity, and water content in the standard substances were performed at weighing. Working standard solutions for calibration curve were prepared by appropriate dilutions of the stock in phosphate buffer (0.05 M, pH=8) and were stable for one month in the refrigerator (5 ±3°C). Fortification standard solutions were prepared by dissolving standard substance in methanol at concentrations 10, 5, 1, 0.5, 0.1, and 0.05 mg/mL. All fortification standard solutions have been stored three months at -18°C.

Samples. Samples were obtained from different manufacturers of the medicated feedingstuffs. Six of commercial feedingstuffs for swine, two for poultry, and two for cattle (calves) were used in the validation. All samples were grinded by centrifugal mill (ZM 200, Retsch) with 1.5 mm sieve.

Extraction. Twenty-five-gramme feed sample was put into a 200 mL Erlenmeyer flask and 100 mL of extraction solvent composed of 25% methanol in citrate buffer (0.05 M, pH 5) was added. Extraction was carried out on horizontal-shaker (KS 501, IKA) for 30 min at 250 rpm. The extract (40 mL) was transferred into tubes and centrifuged (6K15, Sigma) for 10 min at 4,000 x g, at 15°C. The supernatant was then transferred into another centrifuge tube and 15 ml of n-hexane was added. After shaking on horizontal-shaker for 10 min at 250 rpm, the tubes were centrifuged for 10 min at 4,000 x g, at 15°C. The upper organic layer and lipid coating were discarded.

Clean-up. The SPE cartridges were sequentially preconditioned with 4 mL of methanol, citrate buffer, SPE solution (25% acetonitrile in phosphate buffer, pH 8) and citrate buffer. Eight millilitres or 1 mL of the extract were transferred on SPE columns and passed through the cartridges at 1 – 2 mL/min flow using mild vacuum. Columns were washed with 4 mL of citrate buffer, 4 mL of water, 4 mL of 1% ammonium carbonate solution and 4 mL of SPE solution. The cartridges were dried (under vacuum) for 5 min, and tylosin A was eluted with 3 mL of acetonitrile to 6 mL conical vials. The samples were placed in heating block (40°C) and brought up to dryness under gentle stream of nitrogen. The residue was reconstituted in 2 mL of 5% methanol in phosphate buffer, pH 8. After mixing for 1 min (IKA), the solution was filtered through syringe filter (modified cellulose, 0.45 μm, i.d. 15 mm, Macherey-Nagel, Germany) to HPLC vials, which were placed in non-thermostatic autosampler. The samples were stable more than 24 h. Fifty microlitre sample was injected into chromatographic system.

HPLC-UV analysis. The chromatographic system was an Agilent Series 1200 HPLC system (Agilent Technologies, Germany) equipped with degasser, binary pump, autosampler, column oven, and ultraviolet detector with absorbance wavelength $\lambda = 282$ nm. The whole system was controlled by Chemstation B03.02 (Agilent, Germany). Chromatographic analysis were performed with isocratic elution on Zorbax Eclipse XDB – C18 (150 x 4.6 mm, 5 μm) analytical column (Agilent, Germany) thermostated at 30°C. The mobile phase was composed of solution A - methanol and acetonitrile (50:50, v/v), and solution B - triethylamine 70 mM solution adjusted to pH 2.5 with orthophosphoric acid and filtered through cellulose acetate filter 0.45 μm, i.d. 47 mm, Sartorius, Germany). Both phases were mixed on the chromatographic system pump at proportion 40% : 60% A : B and 1 mL/min flow rate applied.

Validation. The whole procedure was checked by evaluation of specificity, linearity, repeatability, and within-laboratory reproducibility (3). To determine the specificity of the method, 12 blank samples of five different types of feedingstuffs for swine and poultry were analysed. The linearity was performed by fortifying blank feed samples to five levels: 5, 10, 50, 100, 500, and 1,000 mg/kg with tylosin A.
Table 1
Validation data of analytical procedure for LC-UV determination of tylosin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>tylosin A</td>
</tr>
<tr>
<td>Linear regression equation (y=ax+b)</td>
<td>y=0.886x-3.4*</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999908</td>
</tr>
<tr>
<td>Working range (mg/kg)</td>
<td>5–1,000</td>
</tr>
<tr>
<td>Limit of detection (mg/kg)</td>
<td>0.87</td>
</tr>
<tr>
<td>Limit of quantification (mg/kg)</td>
<td>2.20</td>
</tr>
<tr>
<td>Level of spiked samples (mg/kg)</td>
<td>10 100 500</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>83.3 82.1 88.8</td>
</tr>
<tr>
<td>Repetitability, CV%</td>
<td>0.25 3.65 2.15</td>
</tr>
<tr>
<td>Reproducibility, CV%</td>
<td>6.51 4.70 2.42</td>
</tr>
<tr>
<td>Uncertainty (mg/kg)</td>
<td>2.1 14.9 24.9</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>21.3 14.9 4.95</td>
</tr>
<tr>
<td>Peak tailing factor</td>
<td>0.96–1.10</td>
</tr>
</tbody>
</table>

* - x – size of the detector signal (peak area).

Fig. 2. Chromatogram (a) blank poultry feedingstuff sample, (b) tylosin A standard at concentration 125 µg/mL, (c) poultry feedingstuff sample spiked with tylosin A at 100 mg/mg, (d) swine feedingstuff sample spiked with tylosin A at 10 mg/kg.
The repeatability was determined by spiking 18 samples at three different concentrations: 10, 100, and 500 mg/kg. The within-laboratory reproducibility was obtained by analysis of two sets of fortified samples at the same concentration levels. The analysis was made with more than two weeks' time interval, by two different operators and with the same HPLC system. The recovery was determined on the basis of analysis of fortified samples with known concentration of analyte.

Results

The typical chromatograms of standard solution, blank, and feedingstuff extracts are shown on Fig. 2. No matrix interference and no interfering peaks from sulfonamids and tiamulin were observed with the retention time of tylosin. Several samples, especially poultry feed samples, gave some interfering peaks near the retention time of tylosin A. Due to the relatively broad peak of tylosin, sum of the areas of all peaks in specific period of retention time was integrated to obtain the true specificity of the method. Although the pH 5 of extraction buffer gave poorer recoveries than alkaline buffers (pH 8 or 9), the obtained variance was significantly lower. The best results were received with a 25% methanol. The SPE SCX (strong cation exchange) cartridges gave cleaner extract than C18 cartridges but higher RSD coefficient, so C18 cartridges were chosen. The volume of sample extract, which was passed through SPE cartridge depended upon sample concentration. When the concentration was higher than 125 mg/kg of tylosin A, 1 ml of extract has been used, in other cases 8 ml. All validation parameters are listed in Table 1.

Discussion

The developed method brought some more satisfactory solutions than procedures recently described in the literature. Generally, sample preparations refer to analyte isolation from biological matrix with methanol (9, 12), acetonitrile (10), or mixture of methanol and buffer solution (8, 11, 18). Tylosin is a weak basis moiety (pKa =7.7) and could be extracted at high pH, but is stable only at pH 4-9. In alkaline pH the tylosin aldol (TAD) and other products are formed, tylosin B is generated in pH below 4. Moreover tylosin exposure to light promotes isotyplosin A formation (14). Extraction with pure organic solvent is nearly 100% efficient but gives numerous impurities peaks and MS detection techniques or longer cleaning procedure would be needed. Evaporation of organic extracts lowers the impurities level but it is extremely difficult due to the presence of lipids. When water was used as an extraction solvent, recovery was lower than 50%, which was not acceptable. Using 25% methanol in citric buffer (pH 5.0) gave the lowest contamination of extract and relatively good recovery. The solution of buffer with methanol at this proportion allowed eliminating evaporation step before solid phase extraction (SPE), and improved defatting, which was necessary due to clogging problems of SPE cartridges.

Previously described methods used the solid phase extraction on C18 (12), HLB Oasis (11, 18), alumina (9, 11), and cyano-propyl cartridges (9). However, more than two steps of SPE (9, 11) significantly increase the time of analysis. It was one of our goals to reduce the sample preparation time and to concise the SPE within one step. The best cleaning process found in the literature was achieved on C18 cartridge (12). In this study, 1% ammonium solution was used in the wash step to remove impurities, which eluted near the retention time of tylosin A, especially in poultry feedingstuffs. The usage of 25% acetonitrile in SPE solution removed most of the impurities without a significant loss of analyte.

Most published methods consider chromatographic separation on C18 or C8 column, using gradient mode of mobile phase. Acetonitrile or methanol, and formic or acetic acid (MS-MS method) (8, 10, 18, 19), or buffer solution at pH 6.3 (11), or pH 2.5 (9, 12) is predominantly used. Tylosin as a weak basis moiety may interact in low pH with free silanol stationary phase groups, causing peak fronting or tailing. In this study isocratic method was optimised on C18 column. The problem of peak symmetry was solved by increasing triethylamine concentration to 0.07 M, which in low pH is ionised and is competing with tylosin to bind the free silanol groups.

The developed method gave good result for 3 g samples prepared in-house, but samples delivered by manufacturers gave considerable high values of RSD. Increasing samples weight to 25 g solved the problem and gave the best results.

In conclusion, the method of tylosin determination in medicated feedingstuffs was successfully developed. The results of validation procedure proved that the presented method is efficient, precise, and useful for routine analysis. The LC-UV analysis and simple sample preparation make the method relatively inexpensive, no time-consuming, and easy to operate. Results presented above allow recommending the method for screening of medicated feedingstuff quality, their homogeneity and stability, which was the main goal of the study, as these parameters determine effectiveness of tylosin therapy.

References


17. http://uphtload.wikimedia.org/wikipedia/commons/d/db/Tylosin.png
