Determination of tylosin residues in pig tissues using high-performance liquid chromatography

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In accordance with the maximum residue limit of 100 \( \mu \text{g kg}^{-1} \) established by EU legislation, a simple and sensitive high-performance liquid chromatographic (HPLC) method was developed for the measurement of tylosin residues in pig tissues (fat, kidney, liver and muscle). Tylosin, a macrolide antibiotic, is extracted with water–methanol and cleaned-up by solid-phase extraction (SPE) on cation-exchange cartridges using methanol–acetonitrile (7 + 3). Tylosin was determined by reversed-phase HPLC with UV detection at 280 nm and the mean recovery from pig tissues fortified in the range 50–200 \( \mu \text{g kg}^{-1} \) was 70–85\%, with intra- and inter-day RSDs in the ranges 3.4–9.1 and 3.9–10.1\% respectively.

**Keywords:** Tylosin residues; pig tissue; cation-exchange cartridge; high-performance liquid chromatography

Tylosin is a macrolide antibiotic which is active against most Gram-positive bacteria and mycoplasmas, but is also active against some Gram-negative organisms and *Chlamydia* spp. Tylosin is widely used in pigs for the control and treatment of swine dysentery (bloody scours) and as a feed additive for promoting animal growth. When used without observing the scheduled dosage and/or withdrawal periods, tylosin can lead to undesirable residues in food of animal origin. EU legislation has established1 a maximum residue limit (MRL) of 100 \( \mu \text{g kg}^{-1} \) for porcine edible tissues. Currently available methods 2–5 for the determination of tylosin in edible pig tissues is needed.

The aim of this work was to develop an extraction procedure and a high-performance liquid chromatographic (HPLC) method for the detection of tylosin residues in all pig tissues for which an MRL had been established (kidney, liver and muscle). However, since tylosin is a lipophilic compound,6 the method was also developed for fat tissue.

According to the guidelines for the approval of analytical techniques for residue analysis,7 methods must be able to determine residues at the MRL and also at half and twice the MRL. To achieve this goal and avoid the use of excessive volumes of chlorinated solvents and laborious clean-up procedures, a selective HPLC analysis coupled with clean-up on a cation-exchange cartridge was adopted.

**Experimental**

**Reagents and standard solutions**

Tylosin (purity as Tylosin A = 97.18\%) and tilmicosin were kindly supplied by Eli Lilly (Indianapolis, IN, USA). HPLC-grade water and organic solvents were obtained from Baker (Deventer, The Netherlands). Reagents were also purchased from Baker and were of analytical-reagent grade. Benzene-sulfonic SCX solid-phase extraction (SPE) cartridges (500 mg per 3 ml) were obtained from Applied Separations (Allentown, PA, USA). Buffer solutions used were 0.1 mol l\(^{-1}\) \( \text{NaH}_2\text{PO}_4 \) (pH 2.5), 0.1 mol l\(^{-1}\) \( \text{Na}_2\text{HPO}_4 \) (pH 4.0) and 0.1 mol l\(^{-1}\) \( \text{Na}_2\text{HPO}_4 \) (pH 9.0). To adjust the pH values, orthophosphoric acid was used. HPLC mobile phase A was 0.01 mol l\(^{-1}\) \( \text{KH}_2\text{PO}_4–0.02 \text{mol l}^{-1}\) tetrabutylammonium bromide adjusted to pH 2 with orthophosphoric acid and mobile phase B was methanol–acetonitrile (7 + 3).

All standard solutions were prepared in methanol–water (2 + 1). A stock standard solution (1 g l\(^{-1}\)) was obtained by dissolving 10 mg of tylosin in 10 ml. Solutions used for sample fortification (10 mg l\(^{-1}\)) were made in 1 tetrabutylammonium bromide adjusted to pH 2 with orthophosphoric acid and mobile phase B was methanol–acetonitrile (7 + 3).

**Apparatus**

The HPLC system (Jasco, Tokyo Japan) consisted of a PU-980 HPLC pump equipped with an LG-980-02 ternary gradient unit, a UV-975 detector and an AS-950 autosampler. A Prodigy analytical column of 5 \( \mu \text{m} \) ODS (250 × 4.60 mm id) (Phenomenex, Torrance, CA, USA) was used. The centrifuge used was a Sorvall Super T 21 (DuPont, Les Ulis, France) equipped with an SL50T rotor. Data handling was performed by Borwin chromatography software (Jasco).

**Sample preparation**

Tissues, collected at a local slaughter house from untreated animals, were first minced using a Waring laboratory blender and then homogenised with an Ultraturrax. The homogenates were stored at \(-80^\circ\text{C}\) pending the extraction procedure. After thawing at room temperature, a 2 g sample was placed in a polypropylene tube. Phosphate buffer solution of pH 2.5 (2 ml) was added to liver and kidney samples in order to improve the recovery of tylosin. This acidification step was unnecessary for fat tissue and for standard injection (25–400 \( \mu \text{g l}^{-1}\) ) were obtained by appropriately diluting the stock standard solution. All standard solutions were stored at \(-80^\circ\text{C}\).

**Sample extraction and clean-up**

To each homogenised sample were added 3 ml of methanol and (with the exception of fat tissue) 15 ml of water. After stirring on a Vortex mixer (30 s) and centrifuging (12 000g for 10 min), the supernatant was transferred into the reservoir connected to the SPE cartridge and water (15 ml) was added to the fat sample extract only. The cartridge had previously been conditioned with 5 ml of methanol followed by 5 ml of phosphate buffer (pH 4). The extract was loaded on to the cartridge at a flow rate of...
1 ml min\(^{-1}\). The cartridge was then washed with 4 ml of water followed by 2 ml of phosphate buffer (pH 9). Before tylosin elution, 0.4 ml of methanol was applied to the cartridge to displace residual phosphate buffer (washing was immediately stopped after this addition). Tylosin was then eluted with 1 ml of methanol at a flow rate of 4 ml min\(^{-1}\). After adding HPLC-grade water up to a final volume of 1.5 ml, the extract was transferred to a vial for HPLC analysis.

**HPLC procedure**

Isocratic elution with the mixed mobile phase [A–B (1 + 1)] at a flow rate of 1 ml min\(^{-1}\) was adopted, using an injection volume of 50 \(\mu\)l. The run time was 12 min, with a post-time of 3 min. Tylosin was detected at 280 nm.

**Validation procedure**

Linearity of the detector response was determined with tylosin standard solutions over the range 25–400 \(\mu\)g l\(^{-1}\). Selectivity was checked by comparing the chromatograms of blank and spiked samples obtained with the reported procedure; the retention time of tilmicosin, a structurally similar macrolide licensed for veterinary use, was also verified.

Repeatability of the recovery assay was determined by extraction in sextuplicate (intra-day) and quintuplicate (inter-day) of each of the four tissues, fortified at either 50, 100 or 200 \(\mu\)g kg\(^{-1}\). Data from the inter-day assay were also used to test the linearity of the method. Daily performance was checked by analysing blank samples carried through the entire procedure simultaneously with spiked samples.

**Results**

Tylosin eluted with a retention time of 6.25 ± 0.2 min. The retention time of tilmicosin (checked in order to verify the selectivity of the method) was about 3 min. The calibration curves were linear over the entire concentration range (25–400 \(\mu\)g l\(^{-1}\)) with a correlation coefficient always > 0.999. Tylosin extracted from all tissues eluted free of interferences; typical blank and spiked samples chromatograms obtained from liver and muscle are shown in Figs. 1 and 2.

Data on the intra-day \((n = 6)\) and inter-day \((n = 5)\) repeatability for each tissue at each spiked concentration level are reported in Table 1. The linearity of the method is shown in

![Typical chromatograms of (a) pig liver spiked with 100 \(\mu\)g kg\(^{-1}\) of tylosin, (b) pig liver spiked with 50 \(\mu\)g kg\(^{-1}\) of tylosin and (c) blank pig liver.](image1)

![Typical chromatograms of (a) pig muscle spiked with 100 \(\mu\)g kg\(^{-1}\) of tylosin, (b) pig muscle spiked with 500 \(\mu\)g kg\(^{-1}\) of tylosin and (c) blank pig muscle.](image2)
Table 1 Intra-day (n = 6) and inter-day (n = 5) mean recovery and RSD of tylosin in pig edible tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentration added µg kg⁻¹</th>
<th>Intra-day</th>
<th></th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean recovery (%)</td>
<td>RSD (%)</td>
<td>Mean recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Fat</td>
<td>50</td>
<td>76.01</td>
<td>7.88</td>
<td>77.80</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>73.22</td>
<td>8.47</td>
<td>73.70</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>74.27</td>
<td>6.76</td>
<td>81.39</td>
</tr>
<tr>
<td>Kidney</td>
<td>50</td>
<td>81.30</td>
<td>6.50</td>
<td>84.34</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>83.14</td>
<td>6.67</td>
<td>77.67</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>80.05</td>
<td>9.08</td>
<td>77.65</td>
</tr>
<tr>
<td>Liver</td>
<td>50</td>
<td>81.84</td>
<td>7.24</td>
<td>71.92</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>71.28</td>
<td>6.99</td>
<td>71.82</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>70.20</td>
<td>3.43</td>
<td>66.06</td>
</tr>
<tr>
<td>Muscle</td>
<td>50</td>
<td>89.54</td>
<td>5.08</td>
<td>88.79</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>82.13</td>
<td>7.68</td>
<td>79.08</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>82.56</td>
<td>3.57</td>
<td>87.90</td>
</tr>
</tbody>
</table>

Table 2 Linearity for assay of pig tissues spiked at three concentration levels (50, 100 and 200 µg kg⁻¹) on five different days. Each line was obtained by plotting 15 data points (inter-day assay)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Equation of regression line*</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>y = 74.31x - 294.63</td>
<td>0.9976</td>
</tr>
<tr>
<td>Kidney</td>
<td>y = 68.29x + 374.32</td>
<td>0.9983</td>
</tr>
<tr>
<td>Liver</td>
<td>y = 64.68x + 38.08</td>
<td>0.9955</td>
</tr>
<tr>
<td>Muscle</td>
<td>y = 78.70x - 60.91</td>
<td>0.9972</td>
</tr>
</tbody>
</table>

* Where x is the tylosin concentration (µg kg⁻¹) and y is the peak area (arbitrary units).

Table 2, where equations for the regression lines and correlation coefficients for each tissue analysed are reported. For all the matrices analysed the intercept was not statistically different from zero. In Fig. 3 the regression lines are given together with the calibration plot.

The recoveries calculated as means ± s for five samples at each concentration level (n = 15) were 77.63 ± 5.09% (fat), 79.89 ± 5.92% (kidney), 69.93 ± 6.52% (liver) and 85.26 ± 6.76% (muscle). The limits of detection, calculated as the apparent content corresponding to the value of the mean plus 6.76% (muscle). The limits of detection, calculated as the apparent content corresponding to the value of the mean plus 6.76% (muscle). The limits of detection, calculated as the apparent content corresponding to the value of the mean plus 6.76% (muscle). The limits of detection, calculated as the apparent content corresponding to the value of the mean plus 6.76% (muscle). The limits of detection, calculated as the apparent content corresponding to the value of the mean plus 6.76% (muscle).

**Discussion**

The chromatographic conditions adopted coupled with SPE clean-up on a sulfonic acid cartridge allow the separation of tylosin from any co-extractives. The relatively short run time is suitable for practical control purposes (routine analysis), allowing a run to be completed every 15 min. Interference from tilmicosin, the retention time of which is about half that of tylosin, can be excluded. Preliminary assays showed that the recovery of tylosin from pig liver and kidney progressively decreased when the spiked homogenates were maintained at room temperature pending extraction. As suggested by Moats et al., accurate recoveries from liver and kidney samples could be obtained only by lowering the pH of the homogenate before tylosin addition. A tentative explanation is the occurrence, in liver and kidney homogenates, of residual enzyme activity (inhibited by acidification) responsible for the degradation of tylosin. For this reason, to avoid risk of tylosin degradation in liver or kidney samples, homogenates must be stored at −80 °C and immediately acidified after thawing. During the development of the method, different water–methanol ratios were tested and the results showed that about 15% of methanol was sufficient for adequate tylosin extraction. High speed centrifugation (12 000 g) was then necessary for a good separation of the extract prior to SPE clean-up.

As tylosin is an ionizable compound, the clean-up procedure was performed on an SCX cartridge. HPLC-grade water was added to the purified extract before HPLC injection to approximate the mobile phase composition, avoiding a concentration step. In order to improve the chromatographic performance, an ion-pair mobile phase was prepared by adding tetrabutylammonium bromide. Considering the good precision and accuracy obtained for all the tissues analysed and the complexity of the matrix, the average recoveries may be considered satisfactory. The extraction procedure does not require chlorinated solvents and uses very small volumes of methanol, making the method cheaper and safer than others published previously for tylosin extraction. The feasibility of the extraction clean-up procedure allows an analyst to process up to 12 samples per day whatever the pig tissue to be analysed.

Although data from interlaboratory trials are also necessary for the conclusive validation of the method, the results on repeatability are in good agreement with validation criteria recommended by the EU. The recent EU regulation on tylosin residues has also extended control to skin + fat sample tissue. The present method is able to detect tylosin in pig fat; further studies should also confirm the applicability to skin + fat sample tissue.

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References


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