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To cite this version:
Soòa Tkáčiková, Ivona Kožárová, Dioniz Máté. Liquid chromatography tandem mass spectrometry
determination of maduramycin residues in the tissues of broiler chickens. Food Additives and Contaminants, 2010, 27 (09), pp.1226-1232. 10.1080/19440049.2010.488252 . hal-00597457

HAL Id: hal-00597457
https://hal.archives-ouvertes.fr/hal-00597457
Submitted on 1 Jun 2011
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<th>Food Additives and Contaminants</th>
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<td>21-Apr-2010</td>
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Liquid chromatography tandem mass spectrometry determination of maduramycin residues in the tissues of broiler chickens

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Abstract

Maduramycin is a polyether ionophoric coccidiostat used to prevent coccidiosis in poultry at a prescribed concentration during a certain time interval. Due to a public health concern about the presence of coccidiostat residues in poultry, the aim of the present study was to determine the level of maduramycin residues in the tissues of broiler chickens fed commercially-produced feed containing 5 mg.kg⁻¹ of maduramycin in complete feed throughout the 5 days of the withdrawal period (WP). The residues were investigated by liquid chromatography (LC) coupled with electrospray ionisation (ESI) tandem mass spectrometry (MS/MS). The limit of detection (LOD) and the limit of quantification (LOQ) of the method were 0.3 µg.kg⁻¹ and 0.8 µg.kg⁻¹, respectively. The average recovery based on matrix-fortified calibrations for chicken tissues was 90 %. Maduramycin was found to be rapidly distributed in all tissues. The highest concentrations of maduramycin residues were found in the heart followed by the skin, liver, gizzard, kidneys, and finally, the muscle (thigh and breast). On day 5 of the WP, the residue concentrations of maduramycin did not decline below the LOQ of the method. Our results emphasize the need to establish the maximum residue limit (MRL) for maduramycin to control the levels of its residues in edible tissues from chickens before slaughter.

Keywords: maduramycin, residues, determination, tissues of broiler chickens, mass spectrometry
Introduction

Coccidiostats belong to the group of pharmacologically active substances which are used as feed additives to prevent coccidiosis in poultry and rabbits, and also as a medicine for the treatment of coccidiosis in food producing animals. In commercial production, the main method of controlling coccidiosis is addition of coccidiostats to the feed at authorised levels and observation of the prescribed hygiene requirements. According to the Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September, 2003, on additives for use in animal nutrition, only monensin, salinomycin, lasalocid, narasin, maduramycin, semduramycin (ionophoric coccidiostats), and nicarbazin, robenidine, diclazuril, halofuginone and decoquinate (chemical coccidiostats) are allowed to be used as feed additives in the European Union (EC 2003). Because these substances have been administered to food producing animals for a long time, it is known, that their residues may be present in the tissues of the respective animals and may pose a risk to public health.

To protect the health of consumers, maximum residue limits (MRLs) should be established in accordance with generally recognised principles of safety assessment, taking into account toxicological risks, environmental contamination, as well as the microbiological and pharmacological effects of residues. The system established by the Council Regulation (EEC) No 2377/90 of 26 June, 1990 (EEC 1990), was recently replaced with the Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May, 2009, laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin (EC 2009a). On January 2010 came into force the Commission Regulation (EU) No 37/2010 of 22 December, 2009, on pharmacologically active substances and their classification regarding MRLs in foodstuffs of animal origin which establishes the MRL only for two coccidiostats belonging to the group of polyether ionophoric antibiotics, monensin in bovine from 2 µg.kg⁻¹ in muscle, kidney, milk to 30 µg.kg⁻¹ in liver, and lasalocid in poultry from 20 µg.kg⁻¹ in muscle to 150 µg.kg⁻¹ in eggs (EU 2010).

Maduramycin (Figure 1) is a polyether ionophoric coccidiostat produced by moulds Actinomadura yumaensis authorised to be used to prevent coccidiosis in fattening chickens and turkeys (max. 16 weeks). It is added to the feed in the form of ammonium salt at a minimum/maximum content of active substance in complete feed of 5 mg.kg⁻¹. The withdrawal period is 5 days. In contrast to the established WP for maduramycin and the
acceptable daily intake (ADI) of 1 µg.kg⁻¹ set by Scientific Committee for Animal Nutrition (SCAN) no MRL is still derived (Scientific Opinion of the Panel on Contaminants in the Food Chain, 2008).

The residues of pharmacologically active substances in products of animal origin should be carefully monitored and controlled in accordance with the Council Directive 96/23/EC of 29 April, 1996, on measures to monitor certain substances and residues thereof in live animals and animal products (EC 1996). Several analytical approaches have been used to determine the coccidiostat residues in different biological matrices, however, those methods were suitable for determination of one or more coccidiostats only. Because maduramycin does not possess a useful chromophore, there was a serious lack of suitable methods for its determination in the past. An enzyme-linked immunosorbent assay (ELISA) methods were initially developed to detect maduramycin residues in poultry. Kennedy et al. (1997) developed an ELISA method for determination of the depletion kinetics of maduramycin residues in poultry using polyclonal antibodies. This method had a LOD of approximately 0.02 ng.g⁻¹ in tissue (ng.ml⁻¹ in serum) and a LOQ of 1.0 ng.g⁻¹ in tissue (ng.ml⁻¹ in serum). The authors reported that the antibodies exhibited minimal cross-reactivity with structurally similar ionophoric coccidiostats. Shen et al. (2001) developed ELISA based on immunoaffinity chromatography cleanup procedure for the analysis of maduramycin in broiler chicken tissues. The limits of detection were 1.0 ng.g⁻¹ in muscle, 2.8 ng.g⁻¹ in liver, and 1.5 ng.g⁻¹ in fat.

Another approach of maduramycin detection is modification of maduramycin with a suitable reagent to obtain an ultraviolet (UV) absorbing product. Quantitative analysis was carried out using a combination of derivatisation procedure and LC separation technique. The Directive No 1497/4/1997-100, as the Official method for investigation of feeds in the Slovak Republic, employed derivatisation with dansylhydrazine and UV (224 nm) or fluorescence detection (excitation 224 nm, emission 515 nm). Tavcar–Kalcher et al. (2009) used dansylhydrazine for determination of maduramycin in concentrates, premixes and feeds. The LOD and LOQ were 0.4 and 1.0 mg kg⁻¹, respectively. De Long et al. (2004) utilised a post-column derivatisation with vanillin for determination of maduramycin in feedstuffs and
premixes by liquid chromatography with UV detection at 520 nm. The LOQ of the method was 2 mg kg\(^{-1}\).

The increasing use of highly selective and sensitive techniques, such as mass spectrometry (MS), tandem mass spectrometry (MS/MS) and time of flight mass spectrometry (TOF/MS), together with advances in liquid chromatography brought about other possibilities of determination of maduramycin residues. The current methods for determination of maduramycin residues in biological matrices (muscle, liver, eggs) are the multiresidue methods based on LC-MS/MS technique (Dubois et al. 2004; Olejnik et al. 2009; Shao et al. 2009). The LC-MS/MS technique is a suitable screening method for large-scale analysis due to simple preparation of samples and short separation time on LC and unlike ELISA method shows a high power of selectivity. The LOD for most of the coccidiostats was below 1 µg.kg\(^{-1}\) and the LOQ was below 2 µg.kg\(^{-1}\). Recently, Stubbings and Bigwood (2009) developed a novel rapid multiresidue/multiclass method suitable to screen for the presence of 41 veterinary drug residues, including ionophores, in animal tissues. In the European Union it is recommended to use mass spectrometry for confirmatory purposes owing to increasing specificity of the method and reducing the likelihood of false positive results.

Because maduramycin is widely used for the prevention of coccidiosis in poultry, the objective of this paper was to determine the residual concentration of maduramycin in various chicken tissues in dependence on the day of the WP. To extract maduramycin residues from the examined samples we selected the method utilised by the Community Reference Laboratory (BVL) in Berlin (Bohm et al. 2005) and adopted and validated it in our laboratory according to Commission Decision 2002/657/EC (EC 2002).

**Materials and methods**

*Experimental animals and pre-preparation of the samples*

Fifteen 17-day old broiler chickens (hybrid Ross) with average weight of 0.510 ± 0.032 kg were used in our experiment. The broiler chickens were randomly divided into two groups, an experimental group (n = 12) fed the commercially-produced feed HYD-02-NORM-TYP containing 5 mg.kg\(^{-1}\) of maduramycin in complete feed and a control group (n = 3) fed unmedicated feed HYD-03-NORM-TYP. All broiler chickens had free access to feed and water. The chickens in experimental group were treated in this way until they were 40 days old. On this day (day 0 of the WP), two chickens were slaughtered and the respective samples
of muscle (breast and thigh), heart, liver, gizzard, kidneys and skin were withdrawn and stored at –20°C until analysis. From day 41 (day 1 of the WP), the remaining chickens were fed unmedicated feed only and slaughtered daily (two chickens per day) up to day 5 of the WP. The samples withdrawn were processed as described above. The control chickens were slaughtered and processed on day 5 of the WP. The weight of slaughtered chickens ranged from 2200 g to 3120 g. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic under the Decision No 155/07-221/3.

Chemicals and reagents
Methanol gradient grade, acetonitrile gradient grade and glacial acetic acid pro analysis were purchased from Merck (Germany). Water was purified by reverse osmosis and deionized by Milli-Q Plus system (Millipore, France). The standard of maduramycin was obtained from Chemos-group (American Cyanamid Company, USA). Standard stock solution (0.5 mg.ml⁻¹) and working standard solutions (2, 5, 10, 20, 30, 40, 60 ng.ml⁻¹) were prepared in methanol. Stability of stock solution is 6 months when stored under refrigeration at –18°C. Working solutions were prepared fresh prior to use.

Sample preparation
Homogenised sample (2 g) and acetonitrile (10 ml) in a centrifuge tube were placed in an ultrasonic bath for 15 min. Then the tubes were shaken for 30 min on a horizontal shaker and centrifuged for 10 min at 4°C at 3000 r.p.m. The acetonitrile extract was dried under the stream of nitrogen at 55°C and re-dissolved in 5 ml of deionised water. The supernatant was purified using a solid phase extraction (SPE, Strata-X, 200 mg, 6 ml, Phenomenex, USA). Before loading the sample onto a column, the column was conditioned with 3 ml of methanol followed by 3 ml of deionised water. The re-dissolved sample extract was passed through the conditioned column by gravity. The column was then washed with 3 ml of deionised water and dried under vacuum for 5 min. Maduramycin was eluted from column with 5 ml of methanol and eluate was concentrated to 1 ml using nitrogen flow at 40°C in TurboVap (Zymark, Swiss). Concentrated eluate was filtered using 0.2 µm syringe filter (Whatman, England) into an autosampler vial. Samples were cooled before analysis to 10 °C.

Matrix spiking
For spiking we used maduramycin free tissues from the control group. A calculated amount of maduramycin standard in methanol was added to the sample prior extraction and methanol was left to evaporate for about 30 min at room temperature. Then the samples were analysed as routine samples.

**LC-MS/MS analysis**

Maduramycin was analysed employing a Waters Alliance 2695 liquid chromatograph (Waters Co., Ireland) connected to the MicroMass Quattro Micro triple-quadrupole mass spectrometer (MicroMass Ltd., UK). Electrospray data were acquired using Mass Lynx software and the instrument was operated in a positive-ion mode. High performance liquid chromatography (HPLC) separation was performed on an analytical column Luna C_{18} 150x2 mm, particle size 3 µm (Phenomenex, USA), at 40°C. The mobile phase was methanol/deionised water/glacial acetic acid 900/99/1 v/v/v delivered at a flow rate of 400 µL.min^{-1}. The injection volume was 10 µl. Maduramycin eluted in about 4 min.

The parameters of the instrument were optimised by means of a standard solution of concentration 100 ng.ml^{-1}. The parameters were: ion source temperature of 100 °C, desolvation temperature of 400 °C, capillary voltage of 3.5 kV, cone voltage of 41 V, collision voltage of 55 eV, extractor voltage of 1 V and RF lens voltage of 0 V. Molecules were bombarded with argon atoms with purity 99.999 %. Nitrogen was used as a nebuliser gas at a flow-rate of 600 L.hour^{-1} and a cone gas at a flow-rate of 50 L.hour^{-1} was produced by Domnik Hunter nitrogen generator (Gateshead, England). Triple quadrupole was operated in multiple reaction monitoring (MRM) mode to achieve high sensitivity. The investigated transitions of maduramycin were 939.36 Da → 877.47 Da, 939.36 Da → 895.6 Da, respectively (Figure 2).

**Method validation**

The following validation parameters were determined: linearity, LOD, LOQ, decision limit (CCα), detection capability (CCβ), recovery, specificity, repeatability and robustness. The linearity of the LC-MS/MS response was proved with 8 calibration points in the concentration range of 0-60 ng.ml^{-1} (working standard solutions including zero concentration). With each set of investigated samples, the new calibration curve was made. The determination coefficients (R^2) of the standard calibration curves were at least 0.99 (Figure 3). The LOD and
LOQ values were found to be 0.3 µg.kg⁻¹ and 0.8 µg.kg⁻¹, respectively, and CCα and CCβ 0.2 µg.kg⁻¹ and 0.3 µg.kg⁻¹, respectively. They were derived from the calibration curve by measuring each calibration point three times. The average recovery from the spiked blank tissue samples was 90 %. Specificity of the LC-MS/MS response was tested by analysing of maduramycin on the presence of the other polyether ionophoric coccidiostats (monensin, salinomycin, lasalocid, narasin, semduramycin) both in standard solution and spiked matrix. No interferences between these compounds were detected. Repeatability was checked by using six muscle tissues fortified with 100 µg.kg⁻¹ of maduramycin. The coefficient of variation was 14.2 %. Robustness of the method was investigated by checking the influence of the following parameters on the results: the volume of acetonitrile used for the extraction, the volume of methanol used for the elution from the SPE column, the flow rate of mobile phase, the desolvation temperature, nebuliser gas flow rate, cone voltage and collision voltage.

Insert “Figure 3” around here.

Results and discussion

The mean residue concentrations of maduramycin (µg.kg⁻¹) determined in respective chicken tissues, not corrected for recovery, and the standard deviations (SD) of measurements are presented in Table 1. No residues of maduramycin were detected in the tissues from the control group (Figure 4).

Insert “Figure 4” around here.

Insert “Table 1” around here.

According to Table 1, detectable residues of maduramycin were found in all examined matrices throughout the withdrawal period. The hearth contained the highest residue concentrations ranging from 285.8 ± 24.7 µg.kg⁻¹ (day 1 of the WP) to 223.5 ± 5.7 µg.kg⁻¹ (day 5 of the WP). This agrees with the toxicological studies which reported that the primary target
organs of the ionophores are cardiac and skeletal muscles (Pressman et al. 1983; Novilla 1992; Shier and Dubourdieu 1992) as well as peripheral nerves (Novilla et al. 1994). These properties have been most completely described for monensin. Relatively high residues of maduramycin were found in the skin (57.7 ± 11.0 µg.kg\(^{-1}\) – 189.7 ± 13.8 µg.kg\(^{-1}\)) which supports the theory that most drugs and medications are lipophilic, tending to deposit in the areas rich in fat, especially in adipose as well as in other organs (Cecchini 2006).

Lower concentrations of maduramycin residues were found in the liver (146.8 ± 34.2 µg.kg\(^{-1}\) – 79.5 ± 3.5 µg.kg\(^{-1}\)) and gizzard (102.0 ± 40.1 µg.kg\(^{-1}\) – 42.1 ± 3.0 µg.kg\(^{-1}\)). The lowest concentrations of maduramycin residues were found in the muscle (15.3 ± 0.7 µg.kg\(^{-1}\) – 11.7 ± 1.1 µg.kg\(^{-1}\)) and kidneys (11.4 ± 1.2 µg.kg\(^{-1}\) – 22.1 ± 4.8 µg.kg\(^{-1}\)).

Because no MRL has been established for maduramycin in edible chicken tissues, our evaluations regarding the food safety issue were based on MRL of 400 µg.kg\(^{-1}\) – 500 µg.kg\(^{-1}\) established for maduramycin in some countries of the world (USA, Canada, Japan). Our results demonstrate that maduramycin at 5 mg.kg\(^{-1}\) of complete feed did not induce residues in the tissues of broiler chickens exceeding the non-EU established MRL. However, detectable residues of maduramycin on the last day of the WP allowed us to state that the establishment of EU MRL for maduramycin in chicken tissues helps to reduce the risk to consumer’s health from the ingestion of maduramycin residues in broiler chickens and thus protects the health of consumers.

It is interesting that we failed to observe a gradual decline in residual concentrations of maduramycin during the WP as can be seen in Figure 6. The maduramycin tissue residue levels on the last day of the WP were several times higher than the LOD of the method. According to the Commission Regulation (EC) No 124/2009 of 10 February, 2009, covering the cross-contamination from non-target animals, the maximum concentration of maduramycin in food of animal origin from animal species other than chickens for fattening
and turkeys is 2 \( \mu g.kg^{-1} \) (EC 2009b). Comparison of this level with the results obtained in our study showed that maduramycin residues in all examined samples on the last day of the WP were several times higher. A 5-day withdrawal period for maduramycin should ensure decline of its residues in the tissues of broiler chickens before slaughter to the levels that present no risk to the consumers. This raises a question whether the edible chicken tissues are really free from maduramycin residues after the 5-day withdrawal period has elapsed, especially considering the fact that the present instrumental technique is able to detect the residues at the levels of \( 10^{-9} \). Liquid chromatography coupled with mass spectrometry detection is one of the most powerful analytical tools in residue analysis and, as seen in our study, seems to be a suitable technique for monitoring of maduramycin residues also in edible tissue of broiler chickens.

Conclusions
Determination of maduramycin residues by mass spectrometry ensured good sensitivity and showed a high power of selectivity. The presented LC-MS/MS method is able to detect maduramycin residues in the tissues of broiler chickens at very low concentrations. It includes a short sample extraction with acetonitrile and a minimal sample purification procedure. This procedure enables the analysis of up to 22 samples within one day and can be used for both screening and confirmatory purposes. The method meets all criteria set by the Decision 2002/657/EC and was successfully implemented in the official testing of coccidiostat residues in poultry, rabbit and bovine in Slovakia in 2007.

Acknowledgements
This study was supported by VEGA grant No 1/0658/09.

References


Figure 1. Molecular structure of maduramycin (Scientific Opinion of the Panel on Contaminants in the Food Chain, 2008).
Figure 2. The mass spectra of the parent ion (939.36) and daughter ions (877.47 and 895.6) of maduramycin performed by continuous infusion of 100 ng.ml⁻¹ in a mixture of methanol/water/glacial acetic acid (900/99/1 (v/v/v)) at a rate of 30 µl.min⁻¹ in electrospray positive mode.
Compound name: maduramycin
Correlation coefficient: $r = 0.999348$, $r^2 = 0.999637$
Calibration curve: $114.669 \times x + 9.26441$
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

Figure 3. The calibration curve of maduramycin standard.
Figure 4. MRM chromatogram of a control sample (skin) under the LOQ of the method (the second daughter ion is missing).
Figure 5. MRM chromatogram of a positive liver sample on day 2 of the WP at the level of 42.0 µg.kg⁻¹.
Figure 6. Comparison of maduramycin residues in the examined chicken tissues (µg kg⁻¹) during the 5 days of WP.
Table 1. Mean maduramycin residue concentrations (µg.kg\(^{-1}\) ± SD) detected in the tissues of broiler chickens throughout the 5 days of the WP by LC-MS/MS.

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<th>4</th>
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<td>109.0 ± 6.4</td>
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<td>158.8 ± 10.1</td>
<td>373.3 ± 24.8</td>
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<td>Skin</td>
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<td>55.4 ± 9.3</td>
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<td>124.7 ± 1.0</td>
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