Validation of a liquid chromatography–tandem mass spectrometric method for the quantification of eight quinolones in bovine muscle, milk and aquacultured products

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Abstract

Quinolones are a group of structurally related antibacterial agents. Over the present decade there has been a significant and progressive increase in the use of this class of antibiotics in animal production. As a consequence the increased use of quinolones can promote the resistance of bacteria. To protect the consumers health, Maximum Residue Limits (MRL) have been established in edible animal matrices by the European Union.

A liquid chromatography–tandem mass spectrometric (LC–MS2) method was developed and validated for the simultaneous quantification of eight quinolones at MRL level in bovine muscle, milk and aquacultured products. The studied quinolones were enrofloxacin, ciprofloxacin, sarafloxacin, danofloxacin, oxolinic acid, flumequine, difloxacin and marbofloxacin. The method involved a single solid-phase extraction followed by the analysis of all quinolones in a single chromatographic run using LC–ESI–MS2. Quinine was selected as internal standard.

This paper consists of two parts: the discussion of the analytical method and the discussion of the different validation parameters according to Commission Decision 2002/657/EEC.

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1. Introduction

Quinolones are a group of structurally related antibacterial agents, which are used in human and veterinary medicine. Their general structure consists of a 1-substituted-1,4-dihydro-4-oxopyridine-3-carboxylic moiety combined with an aromatic or heteroaromatic ring (Fig. 1). Quinolones are used in veterinary medicine for the treatment of pulmonary infections, urinary infections and digestive infections [1]. They exert their therapeutic effects by inhibiting DNA gyrase within the bacterial cell. The carboxylic acid at position 3 and the ketone group at position 4 are necessary for DNA gyrase inhibition, whereas substitutions at position 1 and 7 influence the potency and biological spectrum of activity of the drugs [2]. The administration of quinolones to animals, which are destined for human consumption can result in the presence of residues in food products. These residues represent a potential hazard for the consumer and are a concern due to the emergence of drug-resistant bacteria. Over the present decade there has been a significant and progressive increase in the use of quinolones in animal production [3,4]. The European Union has set Maximum Residue Limits (MRL) for quinolones [5], with the aim of minimising the
risk to human health associated with their residue consumption.

For the determination of quinolones in biological matrices several spectroscopic techniques, such as ultraviolet (UV), fluorescence or mass spectrometry (MS) are used in combination with liquid chromatography (LC). Earlier methods used UV almost exclusively [6], but more recent systems use fluorescence detection [6–19]. These procedures are, however, restricted to a limited number of quinolones. Since several years LC with MS detection has been used for confirmatory analysis because this detection method is more sensitive, selective and allows rapid and multiresidue determination in complex matrices and gives structural information [1,4,6,20–24].

In this work a LC–ESI–MS² method was developed allowing the detection of eight quinolones: enrofloxacin, ciprofloxacin, sarafloxacin, danofloxacin, oxolinic acid, flumequine, difloxacin and marbofloxacin. All quinolones were analysed in a single chromatographic run at MRL level in bovine muscle, milk and tissue of aquacultured products. Previous studies only dealt with one matrix or similar matrices. Quinolones could be detected in aquacultured products or chicken tissue or milk, but no extraction and clean-up method was described which could be used for all these matrices. So each matrix required a specific method development. In this paper a simple and rapid extraction and clean-up method was developed for the different matrices bovine muscle, milk and tissue of aquacultured products. An ion trap mass spectrometer was used as identification as well as confirmation method instead of the more commonly used quadrupole mass spectrometer [1,4,20–22]. A validation was performed for each matrix and the validation parameters selectivity, linearity, accuracy, precision and decision (CCl/H9251) and detection limit (CCl/H9252) are discussed.

### Table 1

<table>
<thead>
<tr>
<th>Instrument parameters precursor ion, isolation width, collision energy and mass range of the the LC–MS² method for the detection of quinolones</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Precursor ion, isolation width, collision energy</strong></td>
</tr>
<tr>
<td><strong>Segment 1</strong></td>
</tr>
<tr>
<td>Scan event 1</td>
</tr>
<tr>
<td>Scan event 2</td>
</tr>
<tr>
<td><strong>Segment 2</strong></td>
</tr>
<tr>
<td>Scan event 1</td>
</tr>
<tr>
<td>Scan event 2</td>
</tr>
<tr>
<td><strong>Segment 3</strong></td>
</tr>
<tr>
<td>Scan event 1</td>
</tr>
<tr>
<td>Scan event 2</td>
</tr>
</tbody>
</table>

### 2. Experimental

#### 2.1. Reagents and chemicals

The quinolone standards, enrofloxacin and ciprofloxacin were obtained from ICN Biomedicals (Irvine, CA, USA) while flumequine and oxolinic acid were from Sigma–Aldrich (St. Louis, MO, USA), marbofloxacin from Vetoquinol (Aartrijke, Belgium) and sarafloxacin from DFK-CLO (Melle, Belgium). No standards were available for danofloxacin and difloxacin, therefore the veterinary drugs Advocin (Pfizer, UK) and Dicural 50 mg (Fort Dodge Animal Health, The Netherlands), respectively, were used. All chemicals used were of analytical grade from Merck (Darmstadt, Germany).

Stock standard solutions of 1000 ng µl⁻¹ were prepared in ethanol for enrofloxacin, danofloxacin, difloxacin and marbofloxacin; in HPLC–water for ciprofloxacin and in 0.1 M NaOH for flumequine, oxolinic acid and sarafloxacin. For the preparation of working solutions HPLC–water was used. All standard and working solutions were stored at −20 °C.

#### 2.2. Instrumentation

The HPLC apparatus comprised of a 1100 series quaternary pump and an autosampler of Hewlett Packard (Palo Alto, CA, USA). Chromatographic separation was achieved using a Symmetry C18 column (5 µm, 150 mm × 2.1 mm, Waters, Milford, USA). The mobile phase consisted of a mixture of methanol with 0.1% trifluoroacetic acid (A) and water with 0.1% trifluoroacetic acid (B). A linear gradient was run (20% A for 5 min and increasing to 100% in the next 10 min) at a flow rate of 0.3 ml min⁻¹.

Liquid chromatography–tandem mass spectrometric (LC–MS²) detection was carried out with a ThermoFinnigan LCQ Deca ion trap with electrospray ionisation (ESI) interface in positive ion mode (San José, CA, USA). The MS detector was operated in three segments each divided in different scan events (Table 1), so the quinolones were separated both chromatographically and mass spectrometrically.
2.3. Extraction and clean-up

2.3.1. Bovine muscle/aquacultured products

To an amount of 2 g of minced tissue 100 µg kg⁻¹ quinine was added as internal standard. The quinolones were extracted from the tissue using 20 ml ultrapure water. After mixing and centrifugation (5 min, 5500 rpm) only 10 ml supernatant was used for further clean-up. The clean-up was carried out using an Isolute 500 mg C18 SPE Cartridge (IST International, Mid Glamorgan, UK). The columns were conditioned with 2 ml MeOH and 4 ml water. After application of the extract, the cartridge was rinsed with 2 ml MeOH/water (20:80), 2 ml hexane and vacuum dried. The quinolones were eluted from the column with 3 ml 1% trifluoroacetic acid in acetonitrile. The eluate was evaporated to dryness at 45°C under a stream of nitrogen. The residues were reconstituted in 30 µl methanol with 0.1% trifluoroacetic acid and 120 µl water with 0.1% trifluoroacetic acid before injecting 15 µl on the HPLC column.

2.3.2. Milk

To an amount of 2 ml milk 100 µg kg⁻¹ quinoline was added as internal standard. To precipitate the proteins present in the milk, 2.5 ml trichloroacetic acid (20% in methanol) was added. After mixing and centrifugation (10 min, 5500 rpm) the quinolones were extracted from the supernatant using 10 ml ultrapure water. The entire supernatant was used for further clean-up after mixing and centrifugation (10 min, 5500 rpm). The clean-up was analogous to the one described for muscle and aquacultured products.

3. Results and discussion

3.1. LC–MS² method

Most methods for the detection of quinolones have been designed for the analysis of individual quinolones or for only two or three compounds, although more recently a number of multiresidue methods have been developed. The method described in this paper is a multiresidue method able to simultaneously detect eight quinolones.

Since most quinolones are fluorescent, liquid chromatography with fluorescence detection is mainly used as determination method for routine residue analysis. Fluorescence depends strongly on the pH of the medium. The highest fluorescence is obtained at a pH value from 2.5 to 4.5, whereas the anionic species do not generally show native fluorescence. Marbofloxacin has a poor native fluorescence and therefore has almost exclusively been determined with UV detection. In this paper the more sensitive, specific and selective detection method ion trap mass spectrometry was chosen. Eight different quinolones, in which marbofloxacin, could be determined with this detection method in a single chromatographic run. Most mass spectrometry methods for the identification of quinolones used a quadrupole mass spectrometer that only monitored specific transitions (precursor–product ion) of each quinolone. In this paper an ion trap mass spectrometer was used as identification as well as confirmation method. So the full scan MS² mass spectrum of each quinolone was recorded which gave more structural information.

The standards enrofloxacin, ciprofloxacin, sarafloxacin, danofloxacin, oxolinic acid, flumequine, difloxacin, marbofloxacin and the internal standard quinine were spiked to blank tissue (bovine muscle and shrimp) and blank milk at the MRL concentration of each quinolone (Table 2). Fig. 2 shows the ion chromatograms of the different quinolones in milk. Similar ion chromatograms were obtained for the matrices bovine muscle and shrimp. Fig. 2 shows all quinolones at their MRL concentration.

Table 2

<table>
<thead>
<tr>
<th>Pharmacologically active substance</th>
<th>Animal species</th>
<th>MRL (µg kg⁻¹)</th>
<th>Target tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrofloxacin (enrofloxacin + ciprofloxacin)</td>
<td>Bovine</td>
<td>100</td>
<td>Muscle</td>
</tr>
<tr>
<td>Sarafloxacin</td>
<td>Salmonidae</td>
<td>30</td>
<td>Muscle</td>
</tr>
<tr>
<td>Danofloxacin</td>
<td>Bovine</td>
<td>200</td>
<td>Muscle</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>Bovine</td>
<td>100</td>
<td>Muscle</td>
</tr>
<tr>
<td>Flumequine</td>
<td>Orte</td>
<td>200</td>
<td>Muscle</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>Bovine</td>
<td>400</td>
<td>Muscle</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>Bovine</td>
<td>150</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td>Porcine</td>
<td>150</td>
<td>Muscle</td>
</tr>
</tbody>
</table>

3.2. Specificity

The specificity of the method could be demonstrated by LC–MS² analysis of blank bovine muscle, blank shrimp muscle and blank milk. No interferences were observed after analysis of these blank samples and after analysis of spiked matrices with all eight quinolones.
Quinolones are veterinary drugs with a MRL, so the minimum number of identification points (IP) is set to three. LC–MS<sup>n</sup> precursor ions earn 1 IP and LC–MS<sup>n</sup> product ions earn 1.5 IP [25].

MS<sup>2</sup>-full scan of the pseudo-molecular ion of the quinolones enrofloxacin, ciprofloxacin, sarafloxacin, danofloxacin and difloxacin each showed two product ions; a loss of 18 due to the loss of COO<sup>-</sup> (Fig. 3). Fragmentation of the pseudo-molecular ion <sup>m/z</sup> 262 of the quinolones oxolinic acid and flumequine only showed the product ion <sup>m/z</sup> 244, due to the loss of water (Fig. 4). So 2.5 IP were earned. Therefore the ion with <sup>m/z</sup> 276 in MS-full scan was also used as a precursor ion, so 3.5 IP were earned. The ion with <sup>m/z</sup> 276 is an adduct ion of the pseudo-molecular ion with <sup>m/z</sup> 262. A mass of 14 was added to the pseudo-molecular ion. The origin of this adduct ion is unclear. The addition of mass 14 has not yet been mentioned in the literature. MS<sup>2</sup>-full scan of the ion with <sup>m/z</sup> 276 was obtained in an extra run for the identification of these quinolones (Fig. 5). MS<sup>2</sup>-fragmentation of the pseudo-molecular ion with <sup>m/z</sup> 262. If a sample contains flumequine or oxolinic acid MS<sup>2</sup>-full scan of the ion with <sup>m/z</sup> 276 will be obtained in an extra run for the identification of these quinolones (Fig. 5).
ion m/z 363 of the quinolone marbofloxacin had a typical MS²-mass spectrum with three product ions, m/z 276, 320 and 345 (Fig. 6). In the MS²-mass spectra of all the quinolones the precursor-ion was still clearly present. There was no improvement by increasing the collision energy. This phenomenon could not be explained.

In Table 3 the specific precursor ions, product ions and the IP of each quinolone are summarised.

3.4. Calibration curve

The chromatographic peak areas, used for the quantification were calculated from the extracted ion chromatograms of the most abundant product ions. These product ions are shown in the legend of Fig. 2.

The calibration curves obtained for the spiked bovine muscle, aquaculture and milk samples were linear in the concentration range 1/2 MRL to 2× MRL for the eight quinolones. However, flumequine in shrimp was an exception. The MRL in aquaculture was 600 μg kg⁻¹. This high concentration can cause space charging in the ion trap. A possible consequence is a non-linear calibration curve. Therefore, samples containing flumequine need to be diluted before quantification. The coefficients of determination were higher than 0.98 for bovine muscle, 0.96 for shrimp (except enrofloxacin, 0.91; and difloxacin, 0.94) and 0.97 for milk.
Table 3
Summary of the selectivity criteria of the different quinolones

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Identification points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrofloxacin</td>
<td>360</td>
<td>316, 342</td>
<td>4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>332</td>
<td>288, 314</td>
<td>4</td>
</tr>
<tr>
<td>Sarafloxacin</td>
<td>386</td>
<td>342, 368</td>
<td>4</td>
</tr>
<tr>
<td>Danofloxacin</td>
<td>358</td>
<td>314, 340</td>
<td>4</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>262, 276</td>
<td>244</td>
<td>3.5</td>
</tr>
<tr>
<td>Flumequine</td>
<td>262, 276</td>
<td>244</td>
<td>3.5</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>400</td>
<td>356, 382</td>
<td>4</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>363</td>
<td>276, 320, 285</td>
<td>5.5</td>
</tr>
</tbody>
</table>

3.5. Accuracy

The accuracy of the method was evaluated at the MRL concentration. For samples spiked at a concentration above 10 μg kg⁻¹, the accuracy of a confirmation method should range from 80 to 110% [25]. Five blank samples were spiked at the MRL concentration for each quinoline and for each matrix. All these samples had an accuracy within the permitted range. In Tables 4–6 the accuracies are summarised for the different quinolines in each matrix. In bovine muscle the accuracies lay within the acceptable range 93–110%, in shrimp between 86 and 107% and in milk between 86 and 102%.

3.6. Precision

The precision of the method was evaluated at the MRL concentration. The coefficient of variation (CV) for the repeated analysis of spiked material, should not exceed the level calculated by the Horwitz equation [25]. For mass fractions lower than 100 μg kg⁻¹ the application of the Horwitz equation gave unacceptable high values. Therefore the CV for concentrations lower than 100 μg kg⁻¹ should be as low as possible. In that case 23% was taken as a guideline for the coefficient of variation (CV at 100 μg kg⁻¹ = 23%). So, 30 spikes for each matrix were analysed and their concentration was determined with the calibration curve. The coefficient of variation was calculated and was lower than the permitted CV. In Tables 4–6 the CV’s are summarised for the different quinolines in each matrix. In bovine muscle the CV’s for the different quinolines were lower than 17%; the CV’s of enrofloxacin and ciprofloxacin were even 7%. All the coefficients of variation in shrimp were lower than 18%. In milk very low CV’s were obtained (lower than 11%), except for marbofloxacin (CV 13%) and difloxacin (CV 16%).

3.7. Decision limit (CCᵢ)

The decision limit is the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. The calculated average concentration of the 30 samples used to determine the precision, plus 1.64 times the corresponding standard deviation equalled the decision limit (α = 5%). In Tables 4–6 the CCᵢ’s are summarised for the different quinolines in each matrix.

The CCᵢ of danofloxacin and difloxacin in bovine muscle can seem rather low looking at their MRL-values in Table 2. In these cases bovine muscle was spiked with the lowest MRL concentration of muscle in general. So danofloxacin was spiked at 100 μg kg⁻¹ (MRL porcine) and difloxacin was spiked at 300 μg kg⁻¹ (MRL turkey, chicken).

In the meanwhile a mini-validation was performed for bovine muscle.
3.8. Detection limit (CC₉₅)

The detection capability is the smallest content of the compound that may be detected, identified and quantified.

4. Conclusion

A LC–ESI–MS² multiresidue method was developed that was able to simultaneously identify and quantify eight quinolones in bovine muscle, tissue of aquacultured products and milk. A simple and rapid extraction and clean-up method was used for the three different matrices. All quinolones were detectable at their MRL concentration and lower. Mass spectrometry was chosen as detection method because this detection method is more sensitive and selective than fluorescence apparatus.

The accuracy and precision of the method were demonstrated since all accuracies were present in the permitted range from 80 to 110% and none of the coefficients of variation did exceed the level calculated by the Horwitz equation or 23 for mass fractions lower than 100 μg kg⁻¹. Using the data of the precision measurements, the decision limit and detection limit could be calculated.

The multi-residue method was validated for the identification and quantification of eight quinolones at MRL-level in bovine muscle, shrimp and milk in correspondence with the criteria of Commission Decision 2002/657/EEC.

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References